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The role of Cyclooxygenase-2 in Wound Epithelium formation

INTRODUCTION

The nature of healing of a wound varies substantially among the diverse groups of the animal kingdom. Some organisms are programmed to respond to injuries in a way that restores complete tissue functionality by building an exact replica of the damaged parts. Many other organisms heal wounds by covering up the damaged portions with replacement tissues, which have physical properties that do not match the original (Clark, 1996; Singer and Clark, 1999). Despite this diversity, the basic steps of healing follow a common theme (Bielefeld et al., 2013). All animals, upon detection of an injury first arrange to cover the wound gap and follow it up with activation of cell division, specialisation of cells and remodelling of this new tissue. Such uniformity in the healing process is perhaps due to the conservation of molecular signalling pathways across the invertebrates and vertebrates.

However so, on taking a closer look at the outcome of wound healing, one finds significant differences, as noted above, and these differences must stem from variations in the regulation of the components of the signalling pathways involved. Differential response to injury can be observed not only across taxonomic groups, but also between different stages in the life of a single organism. The ability to heal a wound perfectly generally lies with early developmental stages of most organisms. In mammals, skin repair that occurs post-natally is very different from that seen in early embryonic stages, wherein the skin regenerates to form tissue undistinguishable from the original one (Dudas et al., 2008; Gurtner et al., 2008). Reasons for this phenomenon are associated with the discussions made in the previous chapter on regeneration and inflammation (Chapter 3). A major reason for near-perfect or perfect wound repair in mammalian foetus is the absence of an inflammatory response (McCallion and Ferguson, 1988; Larson et al., 2010). While the immune system has an unarguably crucial role in protecting an organism from the threats posed by an open wound, the cytokines released during inflammation lead the healing process towards scar formation (Verrecchia and Mauviel, 2002; Leask and Abraham, 2004). The demerit of a scar tissue formed at a site of injury is that it provides only partial restoration of structural integrity. The

scar tissue offers less strength as compared to the original tissues (Clark, 1996; Singer and Clark, 1999). The process of cutaneous wound repair as typically seen in mammals has been summarised in Figure 3.1.

In stark contrast to this type of healing seen in higher vertebrates, many animals display healing of small and large wounds without any scar formation. The highest vertebrates capable of scar-free healing of injuries to most of their tissues are the amphibians. As described by Gilbert (2014), Salamander limbs respond to amputation by a covering formed of epidermal cells and devoid of the dermis. This epidermal covering completes on the first day after amputation. Over the next few days, proteases degrade the extracellular matrix lying under the wound epithelium, allowing mobilisation of cells following their dedifferentiation. This is accompanied by a consequent thickening of the wound epithelium into a multi-layered structure now called the Apical Epithelial Cap (AEC). Although called by many as the Apical Epidermal Cap, Christensen and Tassava (2000) rightly suggest use of the term Apical Epithelial Cap. This suggestion is based on observations that the AEC lacks a basement membrane and dermal components which are characteristics of an epidermis (Thornton, 1954; Ruben and Frothingham, 1958; Salpeter and Singer, 1960; Stocum, 1985). The process of scarless wound healing is indispensable for successful regeneration, because further steps of regeneration such as blastema formation and limb patterning depend on it (Campbell and Crews, 2008). Early studies using histochemistry techniques revealed that the AEC has high secretory activity (Singer and Salpeter, 1961; Tassava et al., 1986; Goldhamer et al., 1989; Estrada et al., 1993). Factors released from the AEC towards the underlying mesenchyme are critical for formation and development of the regeneration blastema (reviewed by Stocum, 1995).

This makes us wonder about the differences in mechanisms underlying the scar-free versus scarred wound healing, because if one is able to elucidate the same, it will help us devise therapeutic approaches to reduce or prevent scarring in human wounds and, optimistically, even induce subsequent regeneration on a large scale.

Fortunately, some differences in molecular signalling are known as a result of research being carried out on different models of wound healing. For instance, it is now well established that low levels of TGF-β1 and high levels of TGF-β3 are associated with scarless healing, a characteristic of fetal wounds (Soo et al., 2003; Larson et al., 2010). It is also believed that stabilisation of β-Catenin protein, which is a central part of the developmentally important

Wnt/β-Catenin pathway, can reprogram the adult dermis to behave like neonate dermis and show improved healing characteristics (Bielefeld et al., 2013). One of the major differences between a scarring wound and a non-scarring wound is the composition of the extracellular matrix (ECM). This is because survival and proliferation of cells, their differentiation states and also their size and shape are closely related to, and even dependent on the dynamics of the surrounding matrix (Godwin et al., 2014). Since the ECM is important in tissue repair and regulation of wound healing, proteases which govern the structure of the ECM are able to influence the outcome of healing. Indeed, a number of matrix metalloproteinases (MMPs) bring about regenerative healing. These are upregulated during the formation of the AEC after limb amputation in urodele amphibians (Ferris et al., 2010). These and other factors influence the regenerative capacity of any tissue in an organism. The current investigation was therefore made to understand whether the pro-regenerative effects of cyclooxygenase-2 was due to its interaction, or at least an influence, on such regulatory pathways. The signalling pathways studied herein are introduced below.

Matrix metalloproteinases

As already mentioned above, the ECM is a landscape of macromolecules that not only makes up the tissue architecture, but also influences the behaviour of local cells (Godwin et al., 2014). Key regulators of the dynamics of the extracellular environment are the MMPs. The MMPs constitute a family of zinc-containing proteases (Visse and Nagase, 2003; Newby, 2005; Barrett et al., 2012). Over 20 proteins have been identified in mammals from which some are secreted and some are membrane bound (reviewed by van Hinsberg and Koolwijk, 2008). As a group, the MMPs have wide-ranging effects on the composition of the ECM and can degrade all the protein components therein (Visse and Nagase, 2003; Newby, 2005; Barrett et al., 2012). What makes the MMPs most relevant to regeneration research is their ability to promote scar-free wound healing. MMPs are necessary for successful limb regeneration in urodeles and most likely act through prevention of scarring (Vinarsky et al., 2005). Shortly following amputation, their expression is upregulated in local limb tissue. One of the earliest members to be induced in axolotl wound epithelium is MMP9 (Yang et al., 1999; Satoh et al., 2007).

Gelatinases MMP2 and MMP9 have also been shown important in fin regeneration in telesosts and tail regeneration in geckos, in studies from our lab Rajaram et al. (2016). These highly conserved peptidases appear in tissue regeneration even in the invertebrates. MMPs are required for the regeneration of the foot in Hydra (Leontovich et al., 2000) and

regeneration of the intestine in Sea cucumber (Quinones et al., 2002). It is worth noting that precise control of the activities of these peptidases must be maintained since, as stated by Stevenson et al. (2006), unregulated MMP activity can be a deterrent to regeneration. As a means of endogenously regulating the MMPs, cells produce a group of inhibitor proteins known as the Tissue Inhibitors of Metalloproteinases (TIMPs). This group of proteins, comprising members transcribed from different genes, is also found upregulated in systems of regeneration following amputation (Stevenson et al., 2006). TIMP1 is able to directly inhibit the activity of multiple MMP members (Sternlicht and Werb, 2001). TIMP2 expression is found to go up after amputation of the Zebrafish fin and its expression correlates with that of other MMPs (Bai et al., 2005), possibly to regulate their activity. Previous work from our lab has highlighted the importance of this pathway in fish fin regeneration. Obstruction of fin regeneration by BMP inhibition was found to be mediated through dysregulation of the MMP pathway (Rajaram et al., 2016). Thus, it was attempted to discover whether the detrimental effects of COX-2 inhibition on regeneration of lizard tail was due to an effect on the functioning of the MMP pathway or not.

Fibroblast Growth Factors

The fibroblast growth factor (FGF) pathway has been well recognised for its involvement in embryonic development (Powers et al., 2000; Bottcher and Niehrs, 2005). The FGF family comprises 23 known ligands and 5 receptors, among other downstream signalling proteins (Powers et al., 2000; Bottcher and Niehrs, 2005). A prominent feature of this pathway is the variety of combinations seen in ligand-receptor binding to govern diverse cellular processes. This makes it rather difficult for researchers to study the roles of pathway members using the common genetic tools (Zhang et al., 2006). Signalling by various FGFs govern the process of limb development in vertebrates. It controls the growth and patterning of the limbs from the very initial stages, i.e. bud formation. FGF2, FGF4, FGF8 and FGF10 help in initiation of the limb-bud and in the formation of the Apical Ectodermal Ridge (AER) – functionally distinct structure at the apex necessary for proper limb development (Mahmood et al., 1995; Savage and Fallon, 1995; Vogel et al., 1996; Ohuchi et al., 1997; Xu et al., 1998). It is known that appendage regeneration in vertebrates proceeds through activation of developmental signalling pathways. The FGF pathway is one such pathway at the fore of regeneration. A number of studies have implicated it in the regenerative wound healing response. Tissue repair is shown to be promoted by ectopically applied FGF ligands FGF1, FGF2, FGF4, FGF7 and FGF10 (Abraham and Klagsbrun, 1988; Werner, 1998). Moreover, FGF1, FGF8

and FGF9 produced from retina trigger the process of lens regeneration in *Xenopus* (Fukui and Henry, 2011). During epimorphic regeneration of axolotl limb, FGF2 was found in the AEC (Mullen et al., 1996). In a similar system of *Xenopus* limb regeneration, the AEC showed the presence of FGF8 (Christen and Slack, 1997). Interestingly, some of the ligands are also expressed during non-regenerative healing of mammalian wounds. FGF2 has been implicated in a number of mammalian wound healing models (Shukla et al., 1998, Swift et al., 1999; Werner et al., 1994a; reviewed by Werner and Grose, 2003). The wound surface and its adjacent dermis contained FGF2 in an incision wound model of adult mice (Whitby and Ferguson, 1991). FGFs were also found in humans after burn wounds (Gibran et al., 1994).

Apart from the FGF ligands, research has also addressed the role of their receptors FGFRs in models of healing and regeneration. Receptor expression has been observed in normal and wounded skin in mice (Werner et al., 1992). Regenerating epidermis in rats following burn injuries displayed prominent expression of FGFR1 (Takenaka et al., 1997). FGFR2 expression has been reported in blastemal cells as well as the wound epithelium in newt (Cannata et al., 2001). Signals mediated through FGFR1 and FGFR2 are also involved in nerve regeneration (Grothe et al., 2006). The necessity of FGF2 signalling in scarless wound healing is exemplified by the work of Giampoli et al. (2003) wherein they compared expression of FGF2 in two urodele species with very different rates of regeneration. They observed that the induction of FGF2 coincides with the formation of AEC in both the species, even though the times are different. Taking a cue from studies on urodeles, our lab had initiated work to investigate the role of FGF in lizard regeneration. Pillai et al. (2013) reported that interference with FGFR-mediated signalling hampered formation of the AEC in the amniote model of epimorphosis. With literature showing evidence of cross-talk between the PGE_2 and FGF pathways (Finetti et al., 2008), it was decided to study the effect of COX-2 inhibition on the expression of key FGF members during wound healing in the lizard tail.

Wnt/β-Catenin pathway

One of the very important pathways functioning during development is the Wnt/β-Catenin pathway. It is well-known for its central role in orchestrating multiple processes of development including cell-proliferation, specification, differentiation and apoptosis. The Wnt family comprises the Wnt glycolipoproteins which have a direct influence on cell-cycle events during tissue development (Logan and Nusse, 2004; MacDonald et al., 2009). These secreted ligands, as is classically known, signal through two main routes – a canonical route

dependent on the β-Catenin protein and another non-canonical route independent of β-Catenin. The current study deals with the canonical pathway, wherein binding of a Wnt ligand to a Frizzled (Fzd) receptor causes stabilisation of β-Catenin protein. β –Catenin translocates into the nucleus, where its association transcription factors T cell factor (TCF) Lymphocyte Enhancing factor (LEF) lead to downstream regulation of gene expression. In the absence of Wnt ligands at the receptors, β-Catenin, which is constantly produced, undergoes proteasomal degradation by the APC-Axin complex (Clevers et al., 2006). Downstream effects of this pathway depend on the specific ligand, the receptor subtype on the target cell and also the concentration of the Wnt proteins in the extracellular space. These ligands therefore act as morphogens and display effects at both short and long range (reviewed by MacDonald et al., 2009).

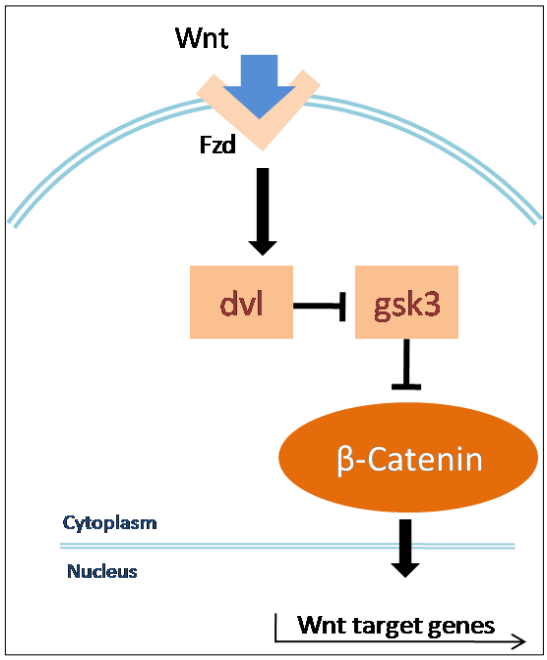

Figure 4.1: Simplified scheme of Wnt/β-Catenin pathway activation

Consistent with its contribution to cell proliferation, fate determination, etc., most genes related to this pathway are active in developing tissues and do not show comparable expression in adult tissues (Caubit et al., 1997a). The pathway is known also for its role in skin development. It regulates development of the dermis and formation of skin appendages (Bielefeld et al., 2013). The development of epithelial structures and dermal components in skin is under the influence of Wnt/β-Catenin pathway (Bielefeld et al., 2013). Even in tissue repair in adults, the Wnt pathway has been widely implicated. For example, the small intestine, which continuously undergoes repair, depends on Wnt signals for its renewal

(Clevers et al., 2014). Following cutaneous wounds in mice, various Wnt ligands are found upregulated over the first few days (Okuse et al., 2005). Mice engineered to maintain elevated β-Catenin levels demonstrated rapid wound healing through collagen deposition, scarring and fibrosis (Kapoor et al., 2008). Since the interest of the lab was to scan for an influence of COX-2 in the process of regeneration, the Wnt pathway seemed to be a meaningful target. Indeed, inhibition of cyclooxygenase by Aspirin or Indomethacin has been earlier reported to reduce signals through β-Catenin (Dilhmann et al., 2001). Goessling et al. (2009) have also identified an association of the two pathways in stem cell and regeneration in Zebrafish. Based on these studies, the Wnt pathway was hypothesised to be a candidate pathway mediating the effects of COX-2 signalling during wound healing in lizard tail.

Transforming growth factors β

In most accounts of cutaneous wound healing, one finds mention of the Transforming growth factor β (TGFβ) pathway. Well-known for its influence on tissue repair, the TGFβ superfamily includes growth factors influencing cell proliferation and differentiation (Wharton and Derynck, 2009). Members of this superfamily, which include TGFβs and the BMPs, among others (Verrecchia and Mauviel, 2002; Owens et al., 2008), exert their effects by binding to receptors TGFβR1 and TGFβR2. This is associated with the activation of Smad proteins, which govern the transcription of various target genes (Verrecchia and Mauviel, 2002, Leask and Abraham, 2004; Owens et al., 2008; Margadant and Sonnenberg, 2010; Biernacka et al., 2011). Three TGFβ isoforms in mammals are known – TGFβ1, TGFβ2 and TGFβ3 – and these play distinct roles in spite of high structural similarities among the three (Moustakas and Heldin, 2009). These growth factors 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). The regulation of TGFβs in cutaneous wound healing has been well documented. Topical administration of this growth factor boosts epithelial closure of wounds in rats (Puolakkainen et al., 1995). Just as in the case of other multi-component signalling systems, the outcome of TGFβ signalling depends on the ligands involved. Participation of different TGFβ ligands is sufficient to decide the course of healing taken by the wounded tissue. TGFβ1, for instance, induces a fibrotic response, leading to scarring of wounds. Topically added TGFβ1 has been shown to improve dermal healing by aiding fibroblast proliferation and matrix deposition in aged rats (Puolakkainen et al., 1995). Infiltration of immune cells and epithelial covering of the wounds was also promoted. The most noteworthy influence of

TGFβ1 is seen on dermal fibroblasts. It induces their proliferation (Schreier et al., 1993; Puolakkainen et al., 1995) and also their production of ECM proteins like collagen and fibronectin (Varga et al., 1987; Hocevar et al., 1999). On the other hand, wounds that heal without forming scars show higher concentration of TGFβ3 and low concentration of TGFβ1 (Soo et al., 2003; Larson et al., 2010).

In view of the close involvement of the above-mentioned molecular pathways in wound healing, the current piece of work was taken up to delineate the molecular mechanism through which COX-2 may result into a scarless and regenerative healing phenotype of the amputation wound. For this purpose, COX-2 activity was abolished using etoricoxib administered to lizards as described in the chapter 2: Material and Methods. After validation of enzyme inhibition (described in chapter 3), tissue segments were collected from the lizards at 4 days post amputation (dpa), the stage when wound epithelium formation is achieved in control animals. Total RNA was isolated and used for cDNA preparation for gene expression analysis. Similarly, protein was isolated from samples and used for protein expression studies by western blots.

MATERIAL AND METHODS

Animals were divided into two experimental groups, *viz.*, control and etoricoxib-treated. Drug administration began at one day prior to amputation (-1 dpa) and continued till wound epithelium stage was achieved (4 dpa). Distal-most segment from the regenerating tail of each animal was collected at the wound epithelium stage and processed for RNA or protein isolation or for immunohistochemistry.

Western blots of MMP2, MMP9, β-Catenin and FGF2 were carried out using 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. The ALP-BCIP/NBT system was employed for colour development.

For immunohistochemistry of β-Catenin, Anti-β-Catenin was used at 3 µg/ml. Detailed protocol has been mentioned in Chapter 2.

Gene expression changes were analysed by quantitative real-time PCR. Results were reported as mean of normalised Cq values in both the experimental groups. Additionally, fold changes

in gene expression was reported for etoricoxib group in relation to the controls (by the method of Livak and Schmittgen, 2001). Primer sequences for all the genes analysed herein have been listed in Chapter 2.

RESULTS

Matrix metalloproteinases

MMP protein expression

Regenerates at wound epithelium stage were screened for changes in protein expression of MMP2 and MMP9. Protein from the regenerating segments of control and etoricoxib treated animals was isolated and 30 µg was used for western blot. Results demonstrate visible decrease in expression of both the tested MMPs in the etoricoxib treated group (Figure 4.2).

Gene expression of MMPs

Gene expression of the MMP genes mmp2, mmp9, mmp14 (or mt1-mmp) and timp2 was tested for any alteration in response to COX-2 inhibition. Real time qPCR analysis was carried out and Cq values of the stated genes were normalised with those of 18S rRNA from the respective samples. Mean normalised Cq values are plotted in Figure 4.3. Higher Cq value reflects lower relative gene expression. Fold change in expression relative to the respective control samples is shown in Table 4.1. A fold change less than 0.5 or greater than 2 was considered as significant to our study. mmp2 and mmp9 expression was significantly reduced in etoricoxib treated group as compared to control ($p<0.001$ and $p<0.05$ respectively). mmp14 also showed a reduction in expression in treated samples, although this was marginally outside the limits of biological significance set by us. timp2, the product of which is a natural inhibitor of MMP activity, showed an increased level of transcripts in response to COX-2 inhibition by etoricoxib ($p<0.05$).

Fibroblast growth factors

Protein expression of FGF2

The expression of FGF2 protein was analysed using western blot. Samples from treatment group regenerates displayed reduced FGF2 protein as compared to control at the wound epithelium stage (Figure 4.2).

Gene expression of FGFs

In order to correlate the change in FGF2 protein content with its transcript, real-time qPCR was used. Mean normalised Cq values of FGF pathway genes expressed in lizard wound epithelium are depicted in Figure 4.4. Expresison of fgf2 transcripts was only 0.44 fold as compared to that in control ($p<0.05$) (Table 4.2). Other ligands of this pathway tested are also shown. Significant up-regulation of transcripts for fgf4, fgf20 and fgf21 is evident in the Cq values as well as in the calculated fold change ($p<0.01$). Genes coding for the FGF receptors which were expressed at this stage were also analysed. No significant change in expression was observed for fgfr2. However, fgfr1 was significantly affected and showed only a 0.06 fold change in expression as compared to control ($p<0.001$), whereas fgfr3 showed an increase at 2.98 fold ($p<0.05$).

Wnt/β-Catenin pathway

β-Catenin protein levels

The level of β-Catenin in regenerates was assessed at wound epithelium stage in control and treatment animals using western blot. Results revealed depletion of stable β-Catenin in etoricoxib treated animals (Figure 4.2), possibly a sign of reduced Wnt signals in the tissue.

β-Catenin localisation

The distribution of active β-Catenin at wound epithelium stage in lizard regenerates has not been documented earlier. Immunostaining was performed on freshly cut frozen sections of 12 µm thickness. Colour was developed by the ALP-BCIP/NBT system. Figure 4.5 is a composite image of micrographs of a longitudinal section of the regenerate. Blue staining is visible mainly along the original plane of amputation.

Wnt ligand expression profile

An expression profile of the Wnt ligands in lizard regenerates was generated using real-time qPCR. To assess the nature of Wnt regulation during the formation of wound epithelium, gene expression of 12 wnt ligands was checked. Of these, transcripts of wnt1, wnt2b, wnt3a, wnt4, wnt6, wnt7a, wnt7b, wnt8a, wnt10a and wnt16 were present in the regenerates. The expression of these genes at wound epithelium stage as relative to resting tail are seen in Figure 4.6. Fold change values have been presented in Table 4.3. Data reveals statistically significant increase in expression of wnt4 and wnt7a at the wound epithelium stage as compared to resting tail tissue.

Gene expression of Wnt ligands

The above-mentioned wnt genes were all tested for any alteration in their expression in response to etoricoxib treatment. Mean normalised Cq values are shown in Figure 4.7. Calculated fold change from Table 4.4 shows a significant decrease in the expression of wnt4 due to COX-2 inhibition ($p<0.001$). Interestingly, 3 of the wnt ligands wnt2b, wnt7a and wnt16 were upregulated in etoricoxib group as compared to control ($p<0.01$, $p<0.05$, $p<0.01$ respectively).

TGFβ ligands

Key ligands of the TGFβ pathway were selected for this study owing to their contribution to the process of wound healing. Research in our lab revealed that tgfβ2 was expressed, although meagrely, at the wound epithelium stage. In etoricoxib group samples, tgfβ2 gene expression went up by 4.7 fold with respect to controls ($p<0.01$) (Figure 4.8; Table 4.5).

DISCUSSION

The process of repair of a cutaneous wound in adult mammals is not entirely different from wound repair in mammalian embryo. The cellular events in both the cases include proliferation, migration, differentiation and apoptosis to repair the damaged tissue (Bielefeld et al., 2013). Despite this, the molecular mechanisms are different since the specific molecular signals differ between embryonic and adult wound healing. It is intriguing that the same set of molecular pathways is able to create two very different molecular environments, guiding the tissues to different fates. As per an important observation made by Wong et al. (2013), there is a greater presence of anti-fibrotic cytokines in a fetal wound, as compared to the adult counterpart.

Scarless healing of wounds is not only a feature of mammalian foetuses, but is also seen in the adults of many organisms. Among the highest vertebrates capable of scar-free healing in adulthood are the lizards. Leopard geckos, for example, show myoblast formation and proliferation of the epidermis and yet, heal without scars (Ud-din et al., 2014). Other lizards noted for their remarkable capacity of perfect healing are the *Hemidactylus* spp. Previous studies on *H. flaviviridis* have highlighted the essential role of the inducible isoform of cyclooxygenase, COX-2. Inhibition of COX-2 activity by pharmacological inhibitor etoricoxib resulted in imperfect healing of the amputation wound in lizard tail (Sharma and

Suresh, 2008). Specifically, negative effects were seen on apoptosis and proliferation of the epidermal cells and subsequent epithelial closure. Since the process of formation and maturation of the Apical Epithelial Cap is concerted by a complex network of molecular signals. It was of interest to explicate which pathways are specifically mediating the negative effects of COX-2 inhibition on healing in amputated lizard tail. For this purpose, candidate molecules from four signalling pathways were selected and their expression levels were analysed in response to COX-2 inhibition by etoricoxib. The dose and its administration are described in Chapter 2: Material and Methods.

Matrix metalloproteinases

The first set of factors selected was the Matrix metalloproteinases (MMPs). This was based on observations of Sharma (2008) that the activity of gelatinases reduced in the healing lizard tail after etoricoxib administration. It remained to be understood whether the reduced activity was due to alteration in their expression or only a post-translational modification of the proteins. Regulation at the level of gene expression was a possibility since one well known decider of their activity is their mRNA levels (Yang and Bryant, 1994; Miyazaki et al., 1996; Yang et al., 1999; Kato et al., 2003; Vinarsky et al., 2005). In the current study, the expression of MMP2 and MMP9 was first tested at the protein level. Western blots showed a clear decrease in expression of both the MMPs in the treated animals. Further, real-time qPCR revealed that this decrease was at the level of mRNA itself. For both the proteinases, their genes mmp2 and mmp9 were significantly down-regulated in response to etoricoxib. Moreover, mt1-mmp, which is a promoter of mmp2 activity, was also down-regulated.

Early expression after amputation is justified as MMPs are known to be active during the inflammatory phase of wound healing (Parks, 1999; Broughton et al., 2006). Some of the substrates of the proteolytic activity of MMP9 include fibrinogen (Lelongt et al., 2001), IL-1 (Ito et al., 1996; Schonbeck et al., 1998) and TGFβ (Yu and Stamenkovic, 2000). It is selective against IL-1β. These are all pro-fibrotic and pro-inflammatory factors. Initiation of limb regeneration is dependent on MMP activity (Vinarsky et al., 2005). These enzymes are indispensable for the processes as their inhibition results in complete failure of regeneration, leaving deformed limb stumps (Vinarsky et al., 2005). Similar observation was made by Ferris et al. (2010) in axolotl wherein MMP9 inhibition prevented epithelialisation of the amputation site.

A major reason that MMP activity has profound effects on regenerative processes is they can liberate growth factors embedded in the extracellular matrix by digesting matrix proteins (van Hinsberg and Koolwijk, 2008). MT1-MMP and MMP2 are both able to degrade a wide range of structural proteins in the matrix (Egeblad and Werb, 2002; Seiki, 2003; Itoh and Seiki, 2006). MT1-MMP itself has many substrates and additionally activates MMP2 to extend its effects (Seiki, 2003; Visse and Nagase, 2003).

It must be noted that the overall outcome of MMPs may not be dependent on their own expression alone, but also on a balance between them and their natural activity inhibitors TIMPs. A number of physiological processes involve the co-expression of MMPs and TIMPs, giving optimal protease action (Gardner and Ghorpade, 2003). In epimorphosis too, timp1 gene is upregulated very early after amputation and remains expressed till late blastema (Stevenson et al., 2006). To identify whether TIMP genes were affected by COX-2 inhibition in lizard during AEC formation, the TIMP isoforms were first screened for their expression. timp2 was found to be expressed during regeneration in the lizard. In zebrafish fin regeneration, timp2 was upregulated and co-expressed with the mt1-mmp and mmp2 (Bai et al., 2005). Interestingly, when COX-2 was inhibited, the expression of timp2 increased as compared to control. This reveals that apart from regulation at gene expression level, the MMPs were inhibited at activity level due to a decreased MMP:TIMP ratio.

Another target of TIMP2 is the FGF pathway. FGF2 signals are inhibited by TIMP2 through inhibition of ERK1/2 (Seo et al., 2008). Also FGF2 can be induced by MMP9, as seen by Ardi et al. (2009).

Fibroblast growth factors

The FGFs are key orchestrators of limb development, as mentioned earlier in this chapter. FGF ligands expressed in the AER of the developing limb bud are responsible for its outgrowth (Niswander and Martin, 1992; Savage et al., 1993; Heikinheimo et al., 1994; Martin, 1998). In regeneration too, FGF2 is seen as essential for AEC formation and maturation, after the work of Pillai et al. (2013). During healing of wounds, fibroblast-derived FGF2 acts on keratinocytes to promote their proliferation and migration (Werner et al., 1994b; Pastar et al., 2014). The current investigation revealed that COX-2 may induce or at least maintain FGF2 expression during AEC formation in lizard. Etoricoxib caused a significant decrease in fgf2 and fgfr1 expression. This positive association of COX-2 and FGF2 could be helping wound healing since FGF2 further enhances genes related to

proliferation and migration (Hughes-Fulford and Li, 2011). An interesting result was the increased expression of fgf4, fgf20 and fgf21 as well as fgfr3 in treatment regenerates. fgf20 expression is known in regenerative cells during the initial stages of fish fin regeneration (Whitehead et al., 2005). While the dysregulation of the expression of the above-mentioned ligands is evident, the significant increase in their expression is difficult to correlate with cellular processes of healing at this juncture. However, the key receptor mediating signals from almost all FGFs is the FGFR1 (reviewed by Thisse and Thisse, 2005) and the expression of this receptor shows heavy setback in treatment group. This observation may suffice in explaining why COX-2 inhibition results in failure of normal regenerative healing in the system under study.

Research shows induction of PGE_2 by FGF2 signalling during osteoblast growth (Hughes-Fulford and Li, 2011). Recent work by Anusree (2012) carried out in *H. flaviviridis* investigated the effect of FGF2 signalling on COX-2 pathway. It was found that FGF2 inhibition had no significant effect on the expression of COX-2 during wound healing in the lizard tail. The present results give reason to believe that PGE_2-induced FGF signalling is also a major possibility in promoting healing. This observation is primed by immunolocalisation experiments (Anusree, 2012) which gave the idea that COX-2 may be an upstream activator of FGF2 signals.

While members of the FGF family are important for early developmental processes such as limb outgrowth, it is their collaborative effect with other key regulatory pathways which make these processes successful. One such closely associated pathway is the Wnt/β-Catenin signalling pathway.

Wnt/β-Catenin pathway

The Wnt/β-Catenin system is attributed with diverse roles such as cell migration, proliferation and wound repair in various animals (Labus et al., 1998; Otto et al., 2008; Wong et al., 2014). β-Catenin has also shown an ability to accelerate carcinogenesis of the mouse cervix (Bulut et al., 2011). It has been associated with malignancy in cervical cancer (Shinohara et al., 2001). As one would expect, tissue regeneration is also vastly dependent on signalling by this pathway. Interfering with Wnt signals caused a heavy delay in re-epithelialisation during mouse endometrial regeneration (Fan et al., 2012). Of course, it must not be overlooked that uncontrolled activity of β-Catenin can be potentially catastrophic for the fate of a healing wound and is believed to be responsible for hypertrophic scars in humans

(Cheon et al., 2005; Sato, 2006). This makes it clear that precise dosage and timing of β-Catenin activity is inevitable for proper scar-free regenerative healing. An alteration therein can be a threat to the success of repair. Western blot from the current work depicts that inhibition of COX-2 leads to decreased presence of stable β-Catenin in the healing wound. Imperfect closure of the wound and improper AEC formation in etoricoxib treated animals (Sharma and Suresh, 2008) can well be attributed to disturbed β-Catenin regulation. Further, it was understood from immunolocalisation that cells in the region of the original plane of amputation expressed β-Catenin. This is the same region which also expressed COX-2 and iNOS, as mentioned in Chapter 3.

To bring to light whether the influence of COX-2-induced PGE_2 on stabilisation of β-Catenin was mediated through the Wnt ligands, the expression profile of these ligands was tested. 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. Among these, wnt4 and wnt7a had significantly greater mRNA levels as compared to an unamputated tail. It can be believed that wnt4 and wnt7a are important for the processes leading to AEC formation. Caubit et al. (1997a) made a similar observation in *Pleurodeles waltl*, where the wound epithelium showed higher level of wnt7a than in the resting tail. All the above-mentioned Wnts were analysed for their expression in response to etoricoxib treatment at the wound healing stage. Wnt4 transcripts dropped heavily due to etoricoxib treatment. It is plausible to relate impaired wound healing to the drop in wnt4 expression since it has been listed as important for timely wound healing and is upregulated post trauma (Labus et al., 1998). Surprisingly, fold change increase in wnt7a in treatment group regenerates was very high compared to control. An exciting set of results recently presented by Ramos-Solano et al. (2015) summarises wnt7a as being detrimental to proliferative activity. Their observations in cervical cancer cell lines form the first report of ectopic wnt7a inhibiting cell proliferation and migration. Further, silencing wnt7a in non-tumorigenic cells led to increased proliferation (Ramos-Solano et al., 2015). A pertinent question now stands – if fold change increase in wnt7a was so high, how can the decrease in β-Catenin protein be explained? The answer may lie in the fact that many factors, other than the Wnts, may control β-Catenin levels during proliferative phase of wound healing (Bielefeld et al., 2011; 2013). Additionally, wnt7a is able to induce both canonical and non-canonical pathways and this

depends on the receptor type involved (Ramos-Solano et al., 2015). The reason of wnt7a having expression at the wound epithelium stage may be related to its role in further blastema formation rather than wound healing. In Axolotl, wnt7a in the regenerating epidermis is implicated in epithelial-mesenchymal interactions (Caubit et al., 1997a).

Transforming growth factor β

Finally, a well-studied regulator of cutaneous wound healing – the TGFβ was tested for alterations in response to etoricoxib. TGFβ ligands were screened in our lab in lizard regenerates at the wound epithelium stage. TGFβ1 and TGFβ2 were expressed very early after amputation. After inhibition of COX-2 enzyme, the expression of TGFβ2 was significantly higher than controls at the wound epithelium stage. TGFβ members are essential for healing of wounds. Mice deficient in TGFβ components were not able to heal dermal wounds perfectly (Ashcroft et al., 1999). Between development and adult wound healing, there is a variation in the TGFβ member involved. While TGFβ1 is seen in adult wounds, it is not important in embryonic stages (Bielefeld et al., 2013). TGFβ3 has been associated with scar-free healing as opposed to TGFβ1 and TGFβ2 (Shah et al., 1995; Soo et al., 2003). When embryonic wounds are administered with TGFβ1, they are led to scar (Lin and Adzick, 1996). TGFβ injections into neonate mice also led to fibrosis (Roberts et al., 1986). TGFβ3, on the other hand, prevents scarring through its modulation of the extracellular environment (Occleston et al., 2011). Although the lizard amputation wound circumvents scar formation and moves towards regenerative healing, this does not appear to be brought about by TGFβ3 since its expression was not found in this system. A relatively recent observation to this effect has also been made in the leopard gecko, wherein TGFβ3 may not be involved in wound epithelium formation (Metcalfe and Ferguson, 2007; Ferguson et al., 2009). However, COX-2 may assist in keeping the levels of TGFβ2 low to prevent fibrosis, since etoricoxib led to an increase in its expression.

Taking together all the results, it is hypothesised that COX-2 activation leads directly to MMP activation during wound healing, which in turn releases FGFs from the matrix and also maintains their expression in the epidermal tissue. FGF2, signalling through FGFR1 must be responsible for Wnt/β-Catenin activation (Finetti et al., 2008). Moreover, TGFβ2 levels are kept low by COX-2 activity to prevent fibrotic healing. This may be closely related with the immune cytokines mentioned in Chapter 3.

Table 4.1: Relative gene expression data for MMP genes in control and etoricoxib-treated animals at wound epithelium stage.

	Mean normalized Cq values		Fold change in Etoricoxib group regenerates
	Control	**Etoricoxib**	
mmp2	27.36 ± 0.17	30.91 ± 0.33 ***	0.09 ± 0.02 ***
mmp9	23.49 ± 0.18	25.00 ± 0.91 *	0.49 ± 0.25 *
mmp14	27.81 ± 0.61	30.45 ± 2.38	0.60 ± 0.32
timp2	23.60 ± 0.06	22.56 ± 0.23 *	2.10 ± 0.33 *

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 4.2: Relative gene expression data for FGF genes in control and etoricoxib-treated animals at wound epithelium stage.

	Mean normalized Cq values		Fold change in Etoricoxib group regenerates
	Control	**Etoricoxib**	
fgf2	23.54 ± 0.13	24.78 ± 0.40 *	0.44 ± 0.12 *
fgf4	25.71 ± 0.68	23.35 ± 0.33 **	5.33 ± 1.42 **
fgf8	26.44 ± 0.27	25.46 ± 0.80	2.28 ± 1.15
fgf10	28.28 ± 1.36	28.02 ± 0.55	1.29 ± 0.47
fgf12	25.29 ± 0.23	24.54 ± 0.62	1.84 ± 0.75
fgf20	27.51 ± 0.01	25.56 ± 0.01 **	3.85 ± 0.01 **
fgf21	25.57 ± 0.34	23.51 ± 0.04 **	4.17 ± 0.12 **
fgf22	25.46 ± 0.55	25.08 ± 0.24	1.31 ± 0.22
fgfr1	27.89 ± 1.73	33.65 ± 1.02**	0.08 ± 0.05 ***
fgfr2	26.01 ± 0.14	25.36 ± 0.47	1.65 ± 0.52
fgfr3	25.94 ± 0.37	24.36 ± 0.12 *	3.00 ± 0.25 *

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 4.3: Relative gene expression data for Wnt genes in resting (0 dpa) versus wound epithelium stage (4 dpa).

	Mean normalized Cq values		**Fold change in Wound epithelium compared to resting**
	Resting	**Wound epithelium**	
wnt1	26.18 ± 0.08	26.85 ± 0.51	0.63 ± 0.02
wnt2b	25.16 ± 0.01	21.55 ± 0.50 ***	12.18 ± 0.69 ***
wnt3a	26.0 ± 0.13	25.93 ± 0.33	1.05 ± 0.10
wnt4	30.24 ± 0.37	27.99 ± 0.12 *	4.76 ± 0.05 ***
wnt6	30.78 ± 1.65	28.71 ± 0.80	4.19 ± 1.95 *
wnt7a	31.78 ± 0.08	26.28 ± 0.14 ***	45.15 ± 0.09 ***
wnt7b	25.96 ± 0.26	25.49 ± 0.51	1.38 ± 0.72
wnt8a	26.14 ± 0.10	26.18 ± 0.30	0.97 ± 0.18
wnt10a	25.95 ± 0.27	24.89 ± 0.39	2.09 ± 0.33 *
wnt16	29.66 ± 0.81	28.63 ± 0.50	2.04 ± 0.09 *

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 4.4: Relative gene expression data for Wnt genes in control and etoricoxib-treated animals at wound epithelium stage.

	Mean normalized Cq values		Fold change in Etoricoxib group regenerates
	Control	**Etoricoxib**	
wnt1	26.85 ± 0.51	26.46 ± 0.80	1.31 ± 0.74
wnt2b	21.55 ± 0.50	18.77 ± 0.17 **	6.87 ± 2.22 **
wnt3a	25.93 ± 0.33	27.18 ± 0.23	0.42 ± 0.07 *
wnt4	27.99 ± 0.12	34.04 ± 0.91 ***	0.02 ± 0.01 ***
wnt6	28.71 ± 0.80	29.33 ± 0.95	0.65 ± 0.64
wnt7a	26.28 ± 0.14	23.65 ± 0.75 *	6.19 ± 4.44 *
wnt7b	25.49 ± 0.51	23.63 ± 0.62	3.65 ± 1.38 *
wnt8a	26.18 ± 0.30	27.09 ± 0.74	0.53 ± 0.38
wnt10a	24.89 ± 0.39	21.85 ± 0.13 **	8.19 ± 0.70 **
wnt16	28.63 ± 0.50	24.58 ± 0.30 **	16.64 ± 3.84 ***

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 4.5: Relative gene expression data for TGFβ2 in control and etoricoxib-treated animals at wound epithelium stage.

	Mean normalized Cq values		Fold change in Etoricoxib group regenerates
	Control	**Etoricoxib**	
tgfβ2	29.94 ± 0.75	27.71 ± 0.49 **	4.96 ± 1.62 **

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.

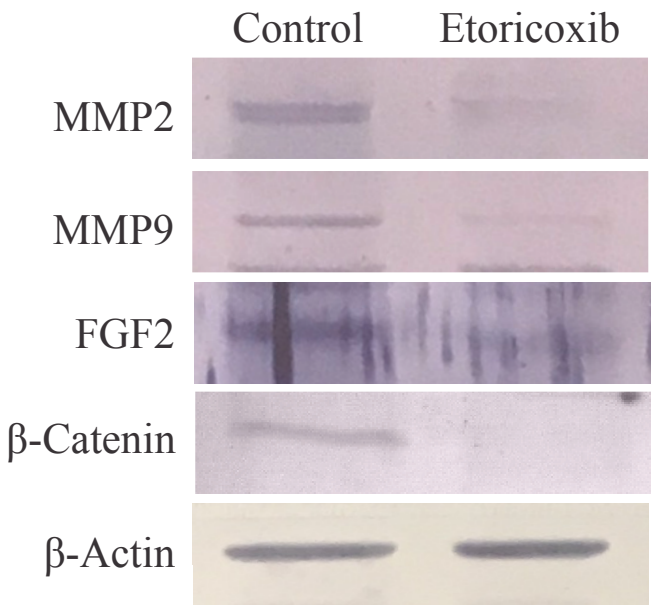

Figure 4.1: Western blot showing expression of various proteins in control and etoricoxib group animals at wound epithelium stage. β-Actin was used as internal loading control.

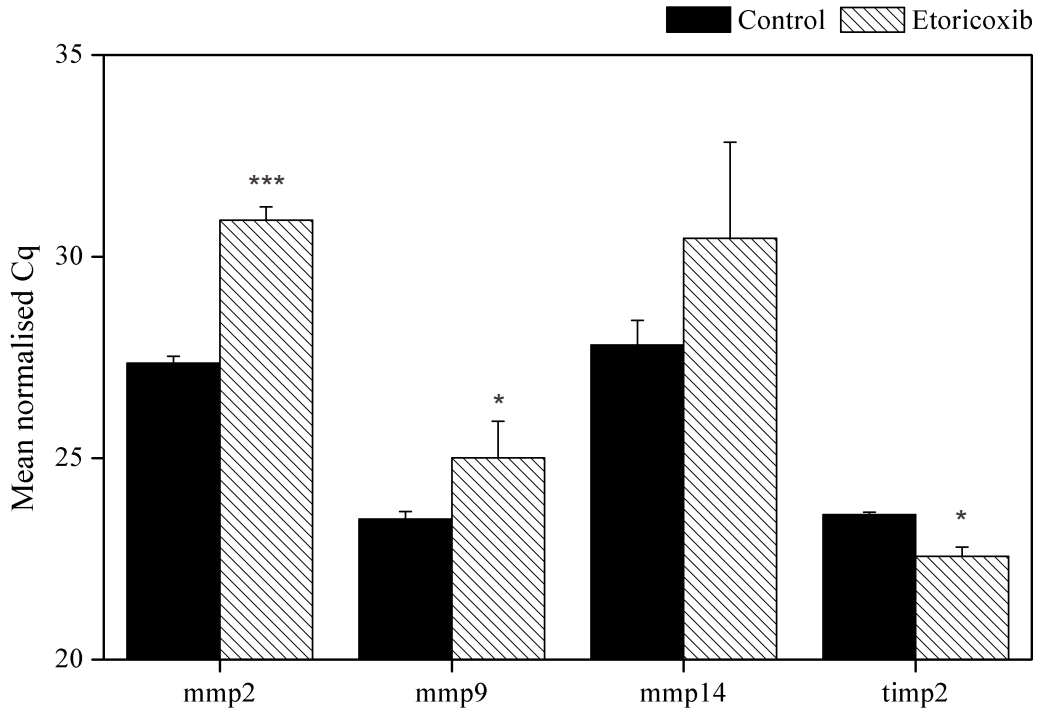

Figure 4.2A: Mean normalised Cq values for MMP genes in control and etoricoxib group animals at wound epithelium stage. Cq value is inversely related to gene expression levels.

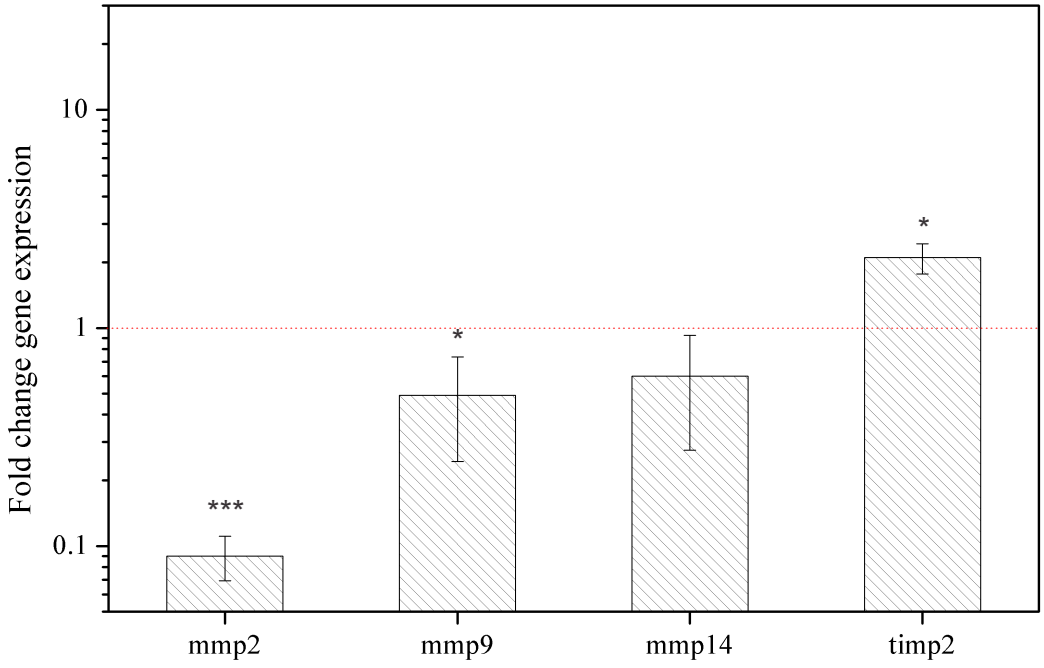

Figure 4.2B: Mean fold change ($2^{-\Delta\Delta Cq}$) in expression of MMP genes in etoricoxib group animals with respect to controls at wound epithelium stage. Dashed red line indicates gene expression level of control group samples taken as 1.

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

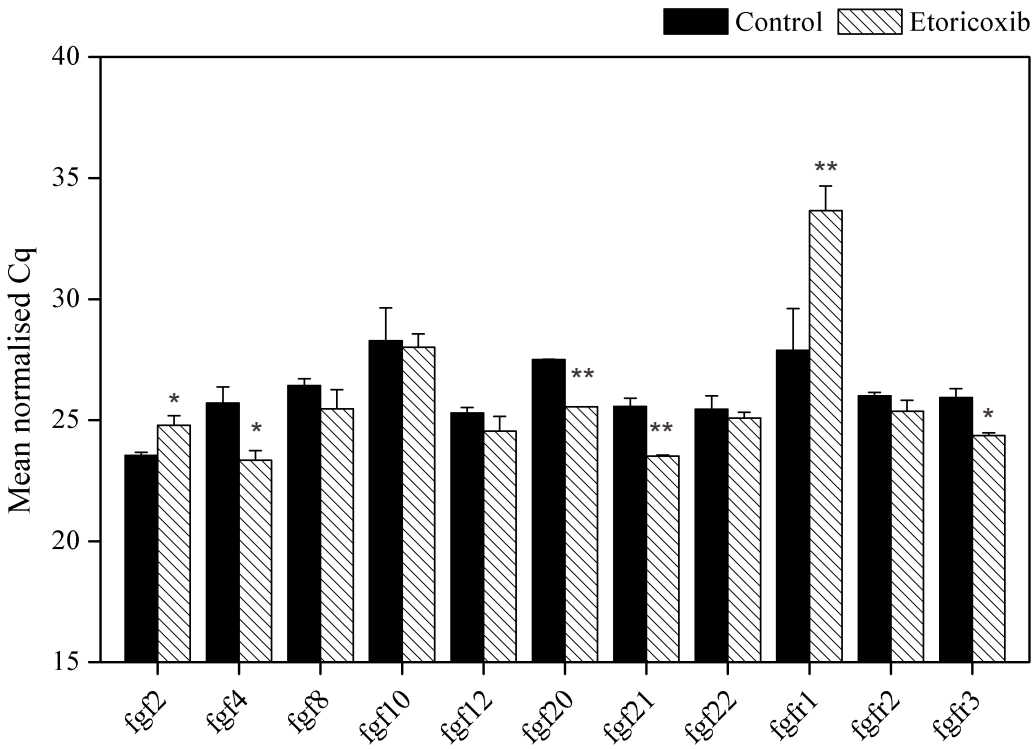

Figure 4.3A: Mean normalised Cq values for FGF genes in control and etoricoxib group animals at wound epithelium stage. Cq value is inversely related to gene expression levels.

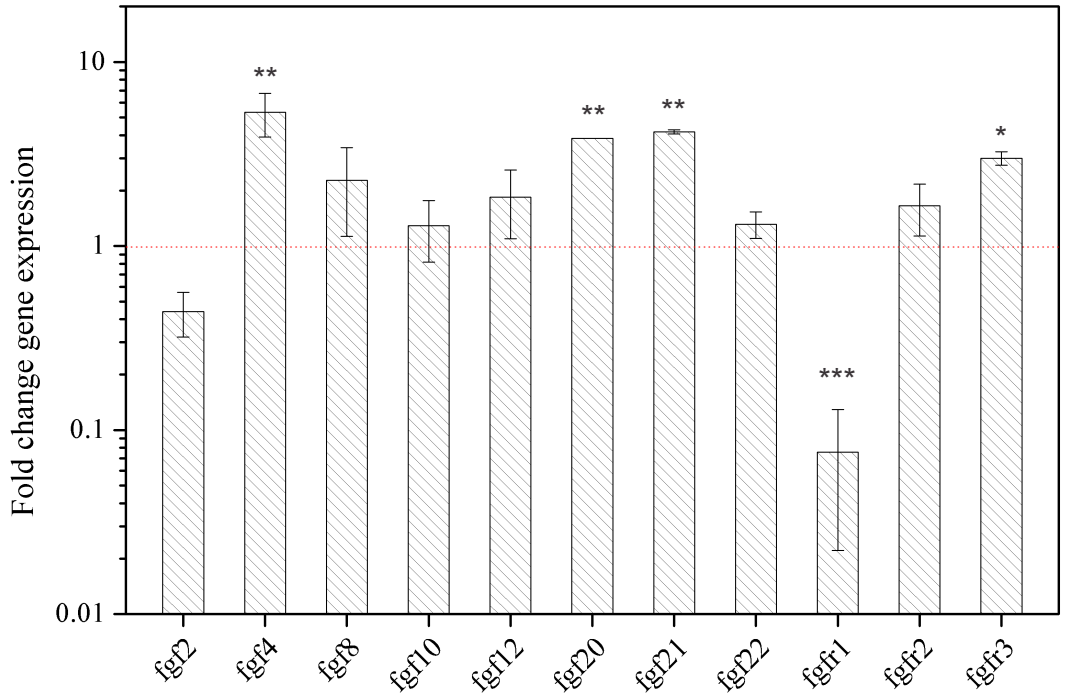

Figure 4.3B: Mean fold change ($2^{-\Delta\Delta Cq}$) in expression of FGF genes in etoricoxib group animals with respect to controls at wound epithelium stage. Dashed red line indicates gene expression level of control group samples taken as 1.

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

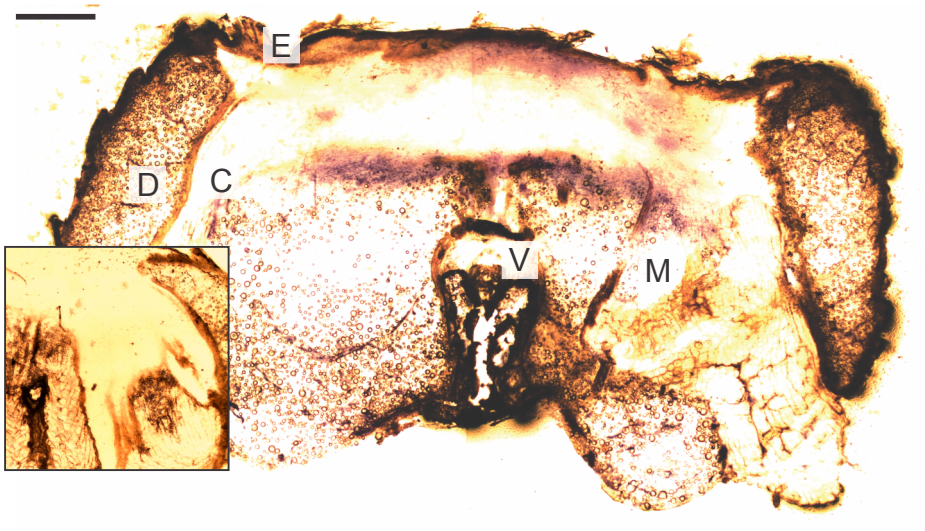

Figure 4.4: Immunohistochemical localisation of β-Catenin in a longitudinal section of wound epithelium stage regenerate. Blue stained region indicates presence of β-Catenin protein. Depicted here is a composite image of multiple micrographs. Negative control for the primary antibody is shown inset. Scale bar represents 500 μm.

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

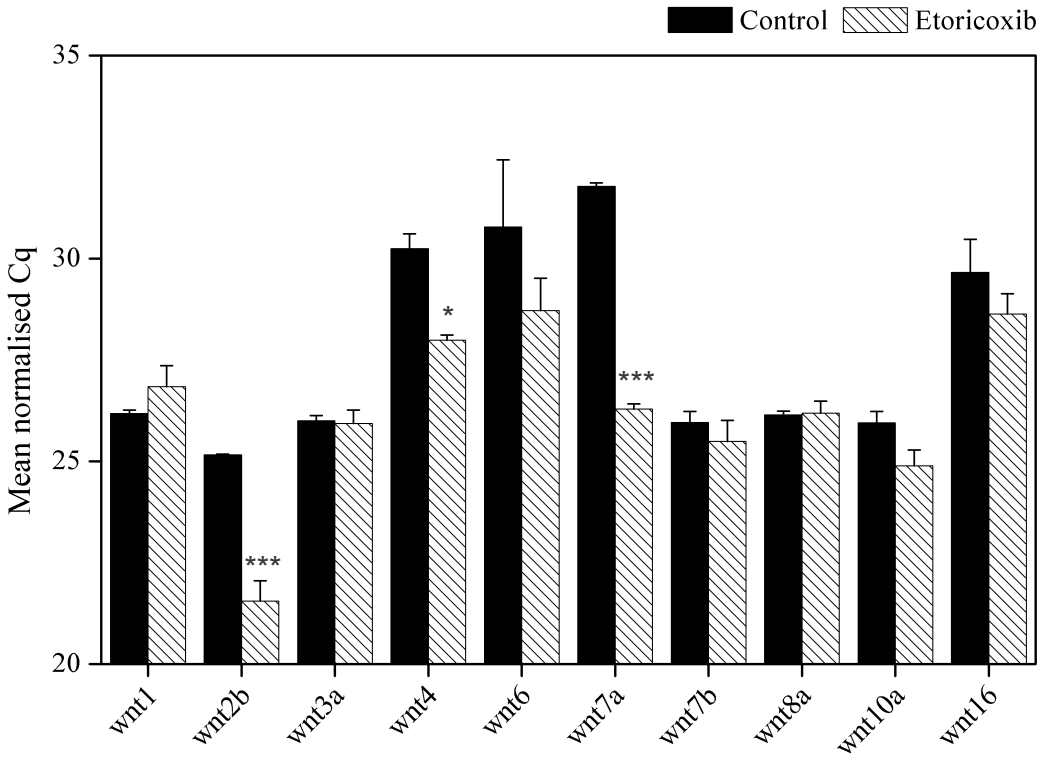

Figure 4.5A: Mean normalised Cq values for Wnt genes in intact (un-amputated) tail and wound epithelium stage regenerates. Cq value is inversely related to gene expression levels.

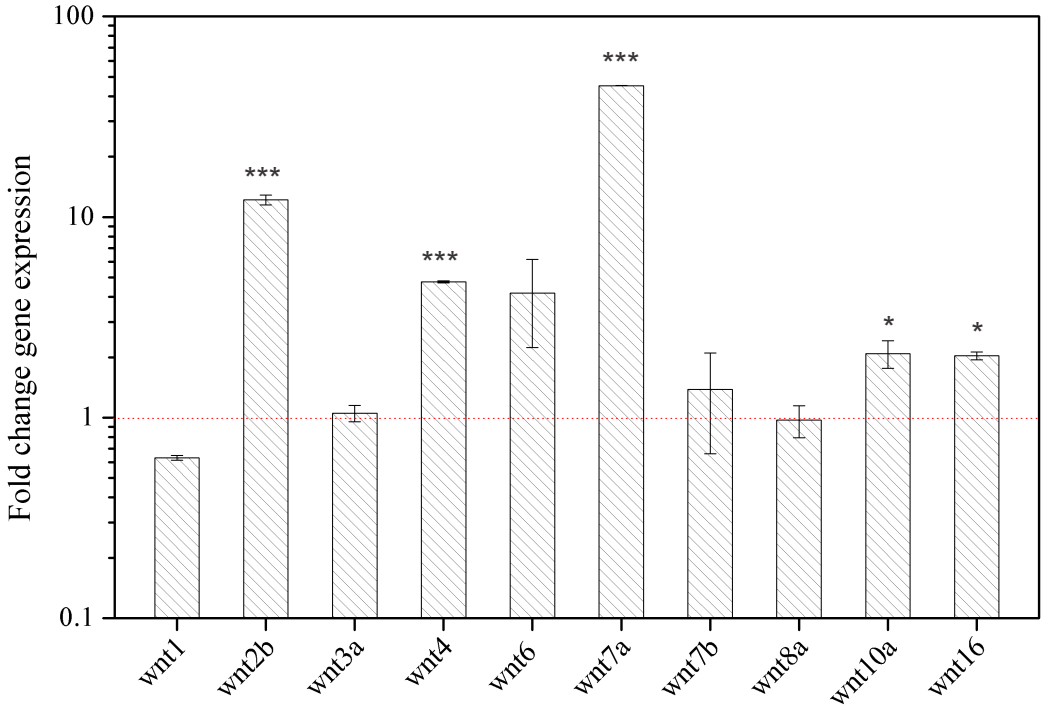

Figure 4.5B: Mean fold change ($2^{-\Delta\Delta Cq}$) in expression of Wnt genes in wound epithelium stage regenerates with respect to intact tail tissue. Dashed red line indicates gene expression level of control group samples taken as 1.

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

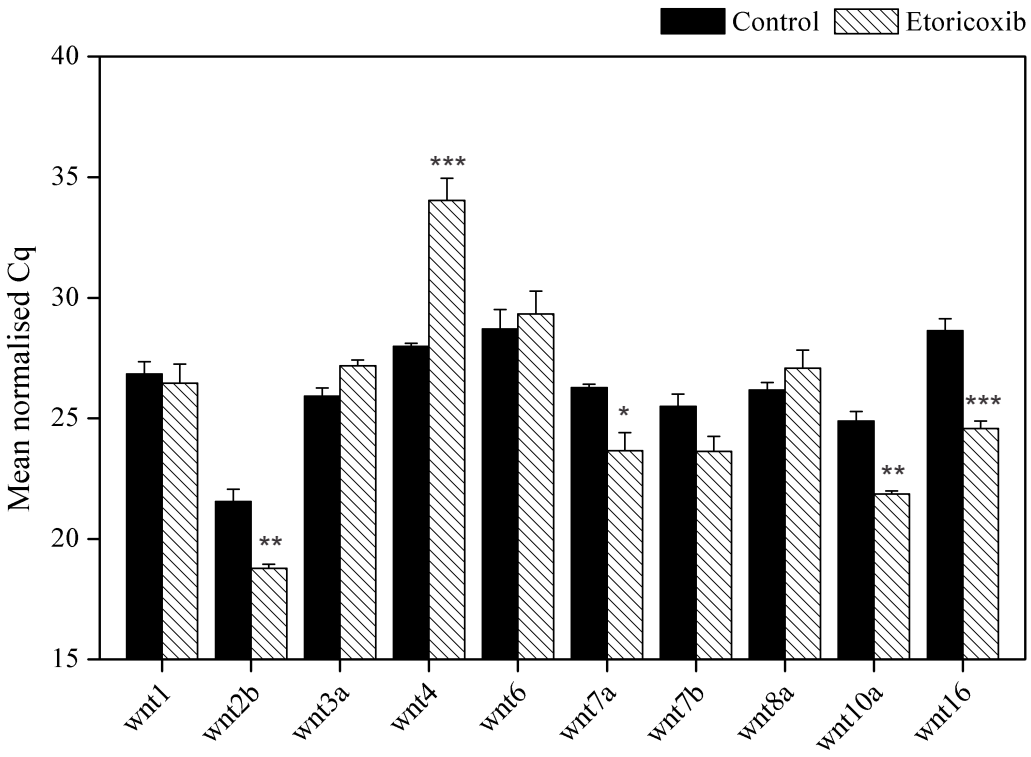

Figure 4.6A: Mean normalised Cq values for canonical Wnt ligand genes in control and etoricoxib group animals at wound epithelium stage. Cq value is inversely related to gene expression levels.

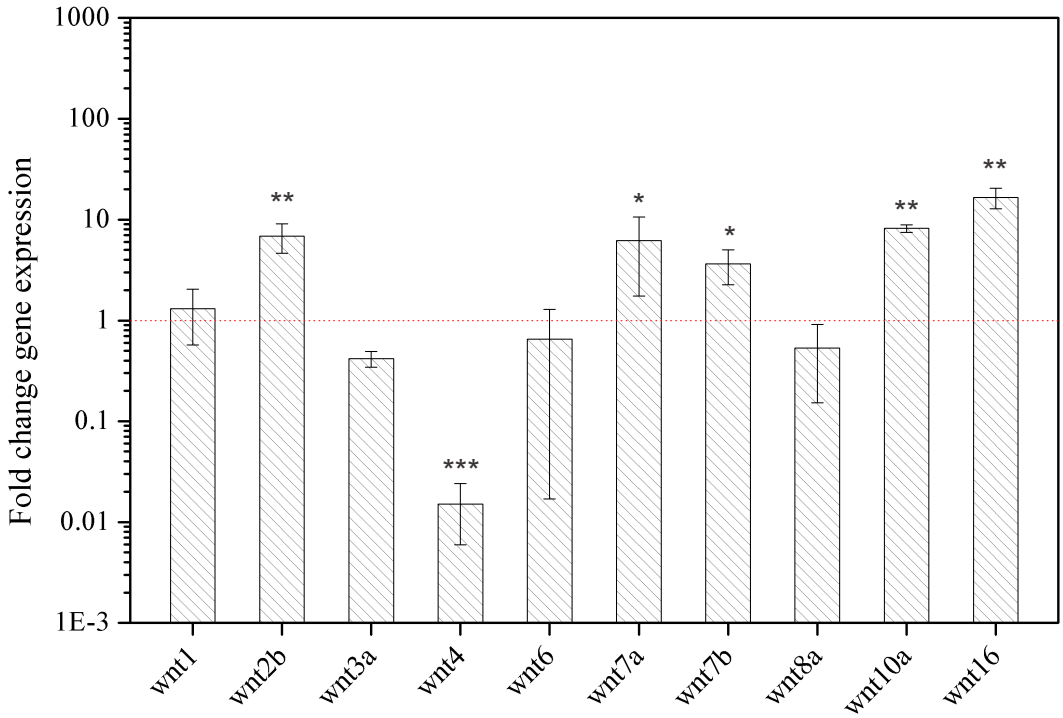

Figure 4.6B: Mean fold change ($2^{-\Delta\Delta Cq}$) in expression of canonical Wnt ligand genes in etoricoxib group animals with respect to controls at wound epithelium stage. Dashed red line indicates gene expression level of control group samples taken as 1.

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

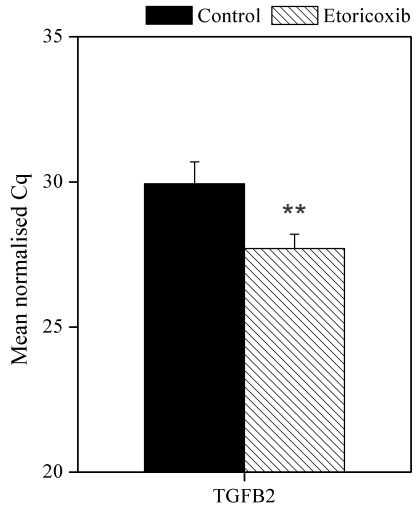

Figure 4.7A: Mean normalised Cq values for TGFβ2 gene in control and etoricoxib group animals at wound epithelium stage. Cq value is inversely related to gene expression levels.

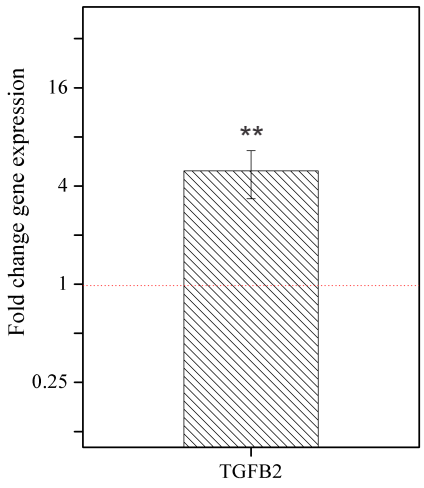

Figure 4.7B: Mean fold change ($2^{-\Delta\Delta Cq}$) in expression of TGFβ2 gene in etoricoxib group animals with respect to controls at wound epithelium stage. Dashed red line indicates gene expression level of control group samples taken as 1.

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