CHAPTER 2

BMP SIGNALING FACILITATES RECRUITMENT OF PLURIPOTENT CELLS DURING EARLY APPENDAGE REGENERATION THROUGH TISSUE ORGANIZATION

INTRODUCTION

Higher vertebrates like mammals have limited ability to regenerate lost organs. Many teleost and urodele amphibians are capable of nearly perfect regeneration of lost appendages. Studies have identified a conserved sequence of events promoting limb and fin regeneration in these animals, which do not take place in the non-regenerative mammalian limb (Brockes and Kumar, 2005; Lovine, 2007; Nakatani *et al*., 2007; Yokoyama, 2008). The fin of teleost is a complex structure where the segmented fin rays (lepidotrichia) are separated by soft inter-ray tissue. Each fin is formed by two concave hemirays, which are lined with osteoblasts that secrete the bone matrix. These hemirays protect an intraray core of mesenchymal cells, blood vessels, nerves, melanocytes, and fibroblasts. The interray space is composed of mesenchymal cells, and an epithelial cell layer covers ray and interray tissue (Tal *et al*., 2010). Adult zebrafish fins in teleost fin regeneration occur through a mechanism known as epimorphic regeneration, whereby a population of mesenchymal cells, the blastema appears at the wound site (Akimenko *et al*., 2003; Schebesta *et al*., 2006). Blastema a mass of dedifferentiated cells is the source of progenitor cells that divide, differentiate and organize for successful restoration of the lost tissue.

The fin of zebrafish is comprised of multiple organized cell types, including osteoblasts, fibroblasts, endothelial cells, neuron and epidermal cells. Each of these cell types regenerates in concert after resection to perfectly restore the entire fin within 2–3 weeks (Gemberling *et al*., 2013). The bony rays, or lepidotrichia, that characterize fish fins are ossified spokes lined with bone forming osteoblasts. Lineage-tracing studies demonstrate that replacement osteoblasts are derived from pre-existing uni-potent osteoblasts (Knopf *et al*., 2011; Sousa *et al*., 2011; Tu and Johnson, 2011; Singh *et al*., 2012; Stewart and Stankunas, 2012), although an unknown population can substitute if needed (Singh *et al*., 2012).

The regenerative ability of an organism also depends on the direct interaction between the wound epithelium and mesenchymal progenitor cells of the blastema. It has been known for decades that contact between the wound epithelium and the underlying blastema is essential for successful regeneration (Chablais and Jazwinska, 2010). However, the underlying mechanisms are understood below par.

Various reports have shown that, after limb amputation, the exposed wound surface is covered by the migrating epidermis/epithelium, called the wound epithelium (Carlson *et al*., 1998; Nye *et al*., 2003; Satoh *et al*., 2008). The wound epithelium interacts with stump tissues, including nerves and then turns into a regeneration specific thickened epithelium called the apical epithelial cap (AEC) (Satoh *et al*., 2008; Satoh *et al*., 2012). Undifferentiated cells are induced by this interaction, accumulated under AEC and form a blastema. Non-regenerative animals consistently fail to show blastema induction after limb amputation. Therefore, blastema induction mechanisms are important to increase the possibility of limb regeneration in higher vertebrates, and they have long been investigated but remain unclear (Makanae *et al*., 2014).

Many signaling pathways, including Activin, BMP, FGF, Sonic hedgehog, Insulin-like growth factor (IGF), Notch, Retinoic acid, Wnt and reactive oxygen species (ROS), are implicated in the regulation of cell proliferation and/or differentiation in non-mammalian vertebrate regeneration (Tal *et al*., 2010; Gemberling *et al*., 2013; Gauron *et al*., 2013; Stewart *et al*., 2014; Wehner *et al*., 2014). In Zebrafish caudal fin regeneration, Wnt/β-catenin signaling indirectly regulates the proliferation of blastema cells, patterning of the epidermis, and differentiation and patterning of bone, mediated through many signaling pathways such as Activin, Notch, FGF, retinoic acid, Hedgehog, IGF and BMP (Wehner *et al*., 2014).

Beck *et al*. (2006) showed that BMP signaling is required for blastema formation, msx1 and FGF-8 expression and proliferation of cells in the epidermis as well as the blastema during *Xenopus* regeneration. In fact, activating msx1 in transgenic frogs amputated during the refractory period stimulates normal regeneration suggesting that msx1 can substitute for BMP signaling in tail regeneration and is likely an important regulator of the mechanism by which BMP signaling stimulates regeneration (Beck *et al*., 2003). Notch signaling inhibition completely abolishes tail regeneration suggesting its requirement during the process. Notch signaling appears to act downstream from BMP signaling (Beck *et al*., 2003).

Blastemal formation is accompanied by extensive remodeling of the extracellular matrix (Calve *et al*., 2010). ECM is composed of a wide variety of components that broadly include proteins, carbohydrates, and proteins modified by sugar moieties termed proteoglycans (PG). Together these components form a complex meshwork that provides both structural and functional information to the cells. Many studies have proved that there is an increase in the activities of a number of types of enzymes that degrade the extracellular matrix during the regressive phase of limb regeneration (Stocum, 1995b; Kato *et al*., 2003). These enzymes include the matrix metalloproteinases (MMPs) (e.g. collagenases, gelatinases, stromelysins), serine proteases (e.g. plasmin), and acid hydrolases (e.g. cathepsin D, acid phosphatase, carboxylic ester hydrolases) thereby altering the organization of extracellular matrix. Similarly, a sweeping change occurs in the extracellular matrix of fin too on amputation and a series of a number of enzymes get activated for the ECM degradation and further growth of fin. Of these enzymes, the matrix metalloproteinases are considered capable of degrading most components of the extracellular matrix (Steteler-Stevenson *et al*., 1993; Nagase and Woessner, 1999). Matrix metalloproteinases have been shown to be involved in remodeling during fin regeneration (Bai *et al*., 2005). More than 25 vertebrate MMPs have been identified so far. MMP contains a zinc-binding motif, and the C-terminal domain plays a role in substrate specificity (Sternlicht and Werb, 2001; Song *et al*., 2006). MMP degradation of structural proteins functions not only to remodel the ECM under physiological situations, but also to degrade tissues under pathological conditions, and release and activate numerous growth factors including fibroblast growth factors (FGFs) and insulin-like growth factor (IGF) (Werb *et al*., 1992; Page-McCaw *et al*., 2007). The spatial and temporal activity of the MMPs is regulated by specific endogenous tissue inhibitors of metalloproteinases (TIMPs). One major function of the TIMPs is to maintain the balance between ECM deposition and degradation and changes in their level can directly affect MMP activity. Numerous studies have demonstrated changes in the expression of various gelatinase and stromelysin transcripts during blastema formation in regenerating urodele (axolotl and newt) limbs (Santosh *et al*., 2011). MMP-2 and 9 are the only two proteases that degrade gelatin (denatured collagen), basal lamina collagens (Type IV, V, VII, X, and XI) and the proteoglycan core protein. MMP-2 and -9, therefore, can breakdown the components of the basement membrane, including laminin, collagen type IV and fibronectin (Yong *et al*., 1998). MMPs may also prevent basement membrane formation under wound epithelium, promoting epithelial/mesenchymal interactions (Yang *et al*., 1999), which are thought to be necessary to support blastemal growth (Tsonis, 1996). A study conducted in our lab showed a reduction in MMP-2 and MMP-9 expression and also impeded wound healing in regenerating fin of teleost fish *Poecilia latipinna* when BMP signaling was blocked (Rajaram *et al*., 2015). Davidson *et al.* (2007) reported elevated extracellular matrix production and degradation upon BMP-2 stimulation which points towards the BMP-2 in cartilage repair and remodeling. Also in many cancer cell lines, the BMP signaling has been reported to inhibit gelatinase expression (Wang *et al*., 2011). Collagen is also known to induce MMPs in breast cancer cells (Thompson *et al*., 1994).

Deposition and remodeling of ECM are essential for regeneration. During the stage of regeneration, infiltrating inflammatory cells and activated resident cells such as fibroblasts and myoblasts deposit a new ECM that provides support for further proliferation and migration of these cells (Grounds, 2008; Serrano and Muñoz-Cánoves, 2010). The continuous proliferation in the blastema causes structure outgrowth by providing new cells, which will differentiate into all the different mesenchymal cell types needed to rebuild the lost body part. The mitotic rate of the blastema slows down as the structure grows, and it ceases completely when the new structure reaches the original size (Wallace and Maden, 1976; Maden, 1977; Smith and Crawley, 1977; Tomlinson *et al*., 1982; Santamaria *et al*., 1996). However, such a complex process requires precise coordination of cell proliferation, cell differentiation, morphogenesis and pattern formation. Cell proliferation is likely to be controlled by a series of specific mitogenic and anti-mitogenic signals, which drive multiple pathways within the cells. One pathway that is likely to be involved in the regulation of blastema cell behaviour is BMP signaling, which previously has been implicated in controlling regeneration in a number of model systems, including mammals (Reddien *et al*., 2007; Guimond *et al*., 2010; Yu *et al*., 2010; Makanae *et al*., 2013; Athippozhy *et al*., 2014). Lehrberg and Gardiner (2015) revealed that in an organotypic sliced model of blastemal cells an optimal dose of human BMP-2 increase the expression of a blastema marker gene (prrx1) and increases the rate of proliferation. Furthermore, incubation with the BMP pathway inhibitor, LDN193189, decreased the rate of proliferation similar to one seen in OSC cultured in the basal medium. In addition, they also reported a similar response (changes in gene expression and proliferation) to signaling from co-cultured nerves, which has been associated previously with FGF as well as BMP signaling (Mullen et *al*., 1996; Makanae *et al*., 2013; Athippozhy *et al*., 2014). The report that an ectopic blastema can be induced by implanting beads soaked in a cocktail of FGF-2, FGF-8, and GDF5/BMP-2/BMP-7 in the absence of a deviated nerve provides additional evidence that blastema formation can be induced by the activation of well characterized and highly conserved signaling pathways (e.g. FGF and BMP) (Makanae *et al*., 2013).

Despite the massive tissue proliferation required during the blastema to regrow the lost organ, an endogenous early apoptotic event is also very crucial for regeneration. Programmed cell death is one such mechanism by which cell number is controlled during morphogenesis (Saunders, 1966; Guha *et al*., 2002). Across phyla, Caspases are known as critical executioners of apoptosis. Although their activity can be deadly, most cells containing these proteins do not die because Caspases are synthesized as inactive zymogens. These proteases gain catalytic activity mostly on activation by upstream apoptotic signals (Thornberry and Lazebnik, 1998). Caspases can be divided into initiator and effector Caspases. Activated initiator Caspases cleave and activate effector Caspases such as Caspase-3, Caspase-7, and Drosophila DrICE and Dcp-1 (Ryoo and Bergmann, 2012).

Tseng *et al.* (2007) reported that cells appear in the regeneration bud of X*enopus laevis* within 12 h of amputation. Surprisingly, when Caspase-3 activity is specifically inhibited, regeneration is abolished. This is true for tails both before and after the refractory period. Programmed cell death is only required during the first 24 h after amputation, as later inhibition has no effect on regeneration. Inhibition of Caspase-dependent apoptosis results in a failure to induce proliferation in the growth zone, a mis-patterning of axons in the regenerate and the appearance of ectopic otoliths in the neural tube, in the context of otherwise normal continued development of the larva (Tseng *et al*., 2007).

In this study, we explored the function of BMP signaling in ECM degradation and cellular activities during blastema stage and also identified the upstream/downstream signaling pathway of the same leading to caudal fin regeneration in teleost fish *Poecilia latipinna.*

MATERIALS AND METHODS

Animals and ethics statements

Sailfin molly, *Poecilia latipinna* (Lesueur, 1821) of both the sexes of the same age, size and weight were purchased from a commercial supplier and maintained under standard conditions of humidity, temperature and light/dark cycle (mentioned earlier in detail). All protocols of handling and processing of fish were carried out according to the ethical principles (Drugs and Cosmetics Rules, 1945) approved by the Institutional Animal Ethics Committee (IAEC) constituted as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. All operations were performed under the hypothermic condition to minimize suffering.

Drug administration and Experimental procedure

All animals were acclimatized for a week before initiation of the study. They were divided into two groups of 32 each. One-third of the original caudal fin from the distal direction was amputated and undisturbed regeneration was allowed for 24hr. For all the studies, the control group received injections of 1% Dimethyl sulfoxide (DMSO) while the treatment group received an injection of LDN193189 (prepared freshly in 1% DMSO) at 2.5mg/kg body weight. 24hpa the drug (single dose/day) was administered intraperitoneally, using a microsyringe (Hamilton Bonaduz AG, Switzerland) and animals were allowed to regenerate. The tissue was collected 60hpa for various studies.

Morphometric study

The time taken to achieve the blastema stage by an animal after amputation was noted and the variation if any in regenerative growth was measured using a calibrated digital vernier caliper (Mitutoyo, Kawasaki, Japan) and photographs (n=4) were taken every day.

Protein Profile: SDS-PAGE

Regenerating tail fin tissue from each experimental group was collected 60hr post amputation, pooled and homogenized in Tris–SDS lysis buffer with protease inhibitor (Sigma- Aldrich, USA). 10% homogenates were assayed for total protein content by Bradford method (Bradford, 1976). An equal amount of total protein was loaded and separated by SDS-PAGE (Laemmli, 1970) on 10% gels, followed by staining with silver stain. Molecular weights of peptides and the relative intensities of all the bands were determined using Doc-ItLs software (GeNei, Merck, USA). A quantitative comparison was made in all the bands of interest, and the results were expressed in arbitrary units.

Gelatin zymography

Herein, 7.5% SDS-polyacrylamide gels with gelatin (5mg/ml) were used. After electrophoresis, gels were washed with 2.5 % Triton X-100, followed by washing in an incubation buffer and then 18hr incubation with the same at 37 C to allow renaturation of protein. Gels were stained with Coomassie Brilliant Blue R-250 and destained in methanol-acetic acid solution. Active MMPs could be observed as clear bands on a dark background.

Hydroxyproline assay

To estimate the total amount of collagen in the regenerated tissue at 60hpa, the zone of interest was excised and acid hydrolyzed in a pressure vessel for 4hr. Upon completion of hydrolysis, hydrolysates and standards were kept in the oven (60°C) for 8hr for complete evaporation of water. Later citric/acetate buffer and chloramine T solution were added. The tubes were incubated for 20min at room temperature and Ehrlich's solution was added to the tubes, which were then incubated at 60°C in water bath for 15min. The absorbance of the reaction product was read at 550 nm. Sample concentrations were determined from the standard curve (details of the protocol followed are mentioned in materials and method).

Real-time RT-PCR

Total RNA was isolated from regenerating fish fin at 60hpa and was homogenized using TRIzol reagent (Applied Biosystems, USA). One microgram of total RNA was reverse-transcribed to cDNA using a one-step cDNA Synthesis Kit (Applied Biosystems, USA).

Sequences of the primers used for the subsequent real-time PCR were as follows:

Gene	**Forward primer**	**Reverse primer**
dlx5	GGAATGCGGATGGGGGATTT	CCACAGCTGAGCCGAAAAAC
runx2	CTGGTGCCTTTTGGGTTGTG	TCGACACGTCTTGCTTAGCG
mmp2	GTCTCCTGGCTCATGCCTTT	TTTCACCACTTGGCCCTCTC
mmp9	GCCTTCAAGGTGTGGAGTGA	ATCCCCGTGGTCAGCTTTTC
18S rRNA	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGGCTGAAC

Quantitative real-time PCR was performed on a LightCycler 96 (Roche Diagnostics, Switzerland) with the following program: 3min at 95ºC, 45 cycles (each cycle of 10s at

95ºC, 10s at 60ºC and 10s at 72ºC). Gel electrophoresis and melt curve analysis were used to confirm specific product formation. Fold change was calculated using Livak method ($2^{-\Delta\Delta Cq}$) (Livak and Schmittgen, 2001).

Histology

Samples were collected and fixed in 10% formalin for a day. These were processed for paraffin embedding as described earlier. Sections of 10µm thickness were cut, stained with Hematoxylin–Eosin and mounted in DPX. Histological profiles were observed and photo-documented using Leica DM2500 microscope.

In vivo BrdU incorporation and immunolocalization

One-third of the tail fin of fishes in both groups was amputated and at 24hpa, both the animal groups were injected with doses under hypothermic condition. Immediately after dosing, the fishes of both groups were incubated in a water tank containing dissolved BrdU (25µg/l) (Sigma, St Louis, USA). The caudal fin regenerates were collected at 60hpa and the zone of interest was fixed in 4% PFA. The fixed tissue was processed for immunofluorescence as per the protocol described earlier in materials and methods.

Immunohistochemistry of Caspase-3

Whole-mount immunohistochemistry was performed to detect activated Caspase-3 activity at 60hpa. Briefly, the zone of interest was collected 60hpa for IHC and was fixed in 4% paraformaldehyde for 4hr. After fixing, the tissue was washed in PBS for 5min, it was processed as per protocol described earlier in materials and methods.

Statistical analysis

Results are expressed as a mean ± standard error of the mean. Statistical significance was determined by Student's t tests. In all cases, a p-value less than or equal to 0.05 was considered statistically significant.

RESULTS

Morphometric studies

The effect of BMP signaling inhibition was detected visibly in morphometric studies which point towards delay in blastema formation. Usual morphology of blastema was not restored in fins of the LDN193189 treated group animals (Figure 2.1). The blastemal epidermis was thin layered and was found more undulating in the fins of the LDN193189 treated animals in relation to fins of control animals (Figure 2.1). Statistical data also revealed a significant decrease ($p \leq 0.001$) in the regenerative growth of tail fin of LDN193189 treated animals (Figure 2.2).

Validation of BMP signaling inhibition by synthetic inhibitor LDN193189

We first confirmed whether intraperitoneal administration of LDN193189 had successfully hampered BMP signaling in the fish fin during blastema stage of regeneration. Two genes downstream of the BMP signal were selected, and their expression was checked in treated and control fins at wound epithelium stage using real-time PCR. These genes dlx5 and runX2 were found to have a significantly low expression in the treated fins as compared to controls (Figure 2.3), with a fold change of 0.000233 and 0.000339, respectively and hence proving the effectiveness of selected drug in blocking BMP signaling.

Histological profile

Control group animals showed a well developed and keratinized apical ectodermal cap (AEC) which was evidently visible. Additionally, accumulation of dedifferentiated blastemal cells (BL) was also observed in histological profile (Figure 2.4A). On the other hand, in treated group, the apical ectodermal cap was less keratinized with trabecular spaces and poorly formed, which indicated a possible functional impairment (Figure 2.4B). Hence, only limited recruitment of blastemal cells was observed in LDN193189 treated group of animals.

Protein profile of blastema is altered due to LDN193189 treatment

After destaining procedure gel images were digitized and spot densitometry was performed on all the bands using Doc-ItLS software (Genei, India). Using densitometric values, the quantitative comparison was made in all the bands of interest and the results were expressed in arbitrary units, which was calculated by integration of the intensity of each pixel over the spot area and normalized for the gel background. Treated group showed the relatively higher number of intense bands compared to control group (Figure 2.5). In treated group, the protein fraction was enriched with polypeptides having molecular masses (in kDa) of 75.84, 72.61, 64.45 and 59.34 as compared to the control. The treated group also showed an observable reduction of protein at a molecular mass of 59.34kDa with respect to control group (Table 2.1).

MMP-2 and MMP-9 activities are reduced after BMP inhibition

Zymogram analysis revealed a sharp rise in the activities of the gelatinases in response to LDN193189 treatment. Intensities of bands corresponding to pro-MMP-9 and active MMP-9 were modulated in treated group i.e. pro-MMP-9 activity (in arbitrary units) upsurged from 68.67 to 73.1 and active MMP-9 activity also amplified from 66.72 to 97.35 after treatment with the BMPR-1 antagonist (Figure 2.6; Table 2.2). Also, there was a development of pro-MMP-2 and active MMP-2 bands in both the groups. Intensities of bands corresponding to pro-MMP-2 and active MMP-2 was changed in treated group i.e. pro-MMP-2 activity increased from 48.06 to 60.26 and active MMP-2 activity also amplified from 50.12 to 63.16 after treatment with the BMPR-1 antagonist (Table 2.2). The LDN193189 treated animals showed an overall enhancement in MMP levels compared to control group animals at blastema stage.

Further, the influence of BMP signaling in the regulation of gelatinases activity was analyzed at the transcript level. The results of the real-time RT-PCR indicated a distinct decrease in the expression of both mmp2 and mmp9 genes in the samples collected from LDN193189 treated fish (Figure 2.7). The mean fold decrease for mmp2 and mmp9 expression was 0.00038 and 0.00012 respectively, as compared to the controls.

Alteration in biochemical component of ECM at blastema stage

Collagen is one of the components of ECM along with other molecular component and aids ECM for processes like growth, wound healing, and fibrosis. Hydroxyproline is the chief constituent of the protein collagen and plays a crucial role in collagen stability. LDN193189 treated group demonstrated a significant reduction in Hydroxyproline levels ($p \leq 0.05$) compared to control group (Figure 2.8), hence presenting an augmented extracellular digestion in the regenerates of the experimental group of fishes.

Cell proliferation during blastema

By 60hpa of regeneration, outgrowth had noticeably advanced and the blastema had formed. It consisted of a mass of morphologically homogeneous, proliferating cells located distally, beneath an elongated epidermal cap. Cell proliferation during this stage was examined by injecting the animals with BrdU. Comparative analysis of the digital images of the tail fin sections, which were processed immunohistochemically for BrdU, revealed that the proliferating cells are more predominant in the regenerated fin rays of control fishes in relation to that of LDN193189 treated fishes (Figure 2.9A and B). Only a few BrdU-positive cells were observed in AEC in fins of the LDN193189 treated animals compared to control. Cells in the inter-ray tissue also followed the same trend. Besides, the intra-ray tissue inside the lepidotrichia also showed a weak rate of proliferation in the treated fins in relation to controls where the tissue showed a strong presence of BrdU-positive cells (Figure 2.9A and B).

Programmed cell death during dedifferentiation of tissue:

Analysis of Caspase-3 activity revealed that apoptosis increased markedly in the fins of treated group animals compared to controls at 60hpa (Figure 2.10A and B). Additionally, apoptotic activity was found amplified in the wound epithelium and blastemal cells. Moreover, as observed during the wound epithelium stage (chapter 1), the pattern uniformity of Caspase-3 activity was not observed in the fins of treated group animals in relation to the fins of the control group animals. The apoptotic activity was more intense near the ray and less towards the interray in LDN193189 treated group animals while the activity was uniform in the fins of control group animals (Figure 2.10A and B).

DISCUSSION

Amputation of an appendage causes massive trauma and disorganization which is followed by rapid formation of wound epidermis, after which pluripotent stem cells are recruited by dedifferentiation of the pre-existing cells, resulting in the formation of a blastema at around 60hpa in *Poecilia latipinna*. Herein, the effect of LDN193189, on the tissue remodeling and cellular activities leading to blastema formation was studied in *P. latipinna*.

We first tested the validity of our treatment method by subjecting the genes dlx5 and runx2 to expression analysis by real-time RT-PCR. A blockage of the BMP signal would lead to downregulation of these genes (Chen *et al.*, 2012). We did find a significant decrease in the expression of both these genes in the healing caudal fin at 60hpa, confirming the inhibition of BMP signal.

Further morphology and histology based experiments were conducted to check the effect of LDN193189 on the dedifferentiated cell mass of the blastema. A significant decrease in the blastemal outgrowth was noticeable ($p \leq 0.001$) after morphometric analysis which points towards delay in blastema formation. Moreover, histological examination showed poorly formed and a less keratinized apical ectodermal cap which indicated functional impairment. Hence, only limited recruitment of blastemal cells was observed. This could be due to the effect of LDN193189 on Epithelial-Mesenchymal interaction, which is mandatory for the blastema formation. The role of BMP members in epithelial-mesenchymal signaling regulation was revealed in this study (Thesleff, 2003).

As predicted, a number of new peptides were synthesized during blastema stage, and few of them down-regulated. A spot densitometric scan of the PAGE gels revealed a relatively more number of intensified bands in LDN treated group. In treated group, the protein fraction was enriched with polypeptides having molecular masses (in kDa) of 75.84, 72.61, 64.45 and 59.34 as compared to the control. The treated group also showed an observable reduction of protein at a molecular mass of 59.34kDa with respect to control group.

Proteolytic degradation of ECM is the key to inflammatory response, cell-cell and cell-matrix interactions and cell migration leading to the formation of the WE, the structure that provides the signals required for subsequent proliferation (Campbell and Crews, 2008). Degradation of ECM also plays an important role in cell migration towards the underlying mesenchymal layer to form a blastema. The major group of proteolytic enzymes responsible for ECM degradation during processes that require ECM turnover, including regeneration is the matrix metalloproteinases. It has been reported that MMP-9 expression in the epidermis is associated with keratinocyte migration and is not needed for downstream WE function (Yang *et al*., 1999; Satoh *et al*., 2008)

We ultimately intended to screen for changes in the expression and activities of the MMP-2 and MMP-9, which fall in this range of molecular weights, since MMPs are very well-known mediators of tissue remodeling after injury (mentioned in earlier chapter) and play a crucial role in blastema formation as well as further progression of epimorphic regeneration of teleost fish. One of the exclusive activities observed was upregulation in MMP-9 and MMP-2 activities and expression of both, which in turn promoted collagenolysis pointing towards high degradation activity in extracellular matrix components during this stage in

LDN193189 treated group, in relation to control group. The results point towards the delay in upregulation of these enzymes due to which they appeared at 60hpa rather than appearing at 24hpa for ECM degradation and also points towards delayed wound healing. The same genes were downregulated at wound healing stage in LDN treated animals. Therefore it was discovered that the time of onset of MMP-2 and MMP-9 expression is sensitive to BMP signal inhibition, which caused a delay of several hours. The activity of MMPs was very well supported by Hydroxyproline estimation. The collagen content was significantly decreased in LDN193189 treated group. Collagen is one of the molecular components of ECM. It can be a reason behind the poor histology and recruitment of blastemal cells after BMP signaling inhibition.

Recently, ECM has been recognized for its critical roles in maintaining a special local environment for adult stem cells, stem cell niche and ECM degradation involved in stem cell differentiation, activation, and/or release (Chen, 2010; Reilly and Engler, 2010). Degradation of the ECM by proteases would break contacts between ECM molecules and integrin receptors, leading to changes in cell shape and reorganization of the actin cytoskeleton (Juliano and Haskill, 1993). In conjunction with Na^+ influx and H^+ efflux (Adams *et al*., 2007), secondary messenger signals such as inositol trisphosphate (IP3) (Rao *et al*., 2009) and growth factors provided by the AEC (Christensen *et al*., 2001, 2002) this reorganization might activate signal transduction pathways that downregulate phenotype-specific transcription programs and up-regulate programs characteristic of a less specialized state that allows blastema cell migration and response to proliferation and patterning signals (Santosh *et al*., 2011).

It is well known that BMP signaling, with Wnt/β-catenin and FGF signals, is also required for blastema formation. The size of an organ or organism depends mainly on its total cell mass, which depends on both the total number of cells and the size of the cells. Cell number, in turn, depends on the amounts of cell division and cell death. We decided to check the effect of LDN193189 on proliferation activity in the fin of teleost *Poecilia latipinna* during blastema stage by *in vivo* BrdU labeling. Cell proliferation activity was significantly decreased at blastema stage revealed by less number of BrdU-positive cells in LDN193189 treated animals. Additionally, epidermis also showed few BrdU-positive cells. BMP signaling is known to be required for blastema proliferation during regenerative outgrowth (Stoick-Cooper et al., 2007a). Beck et al. (2006) also showed that BMP signaling is required for blastema formation, msx1 and FGF-8 expression, and proliferation of cells in the epidermis as well as the blastema. Apart from that, the pattern of blastema in LDN193189 treated animal was different from control group blastema which also points towards alteration in Shh signaling. The current findings were in accordance with the studies conducted by Smith *et al.*, (2006), where inhibition of BMP activity using the naturally occurring BMP antagonist, noggin, showed a reduction in blastema cell proliferation and a reduction in bone matrix deposition.

Regeneration is a complex process possessing a number of distinct phases (Gardiner *et al.*, 2002). Along with cell proliferation, it is also known that certain degree of cell death by apoptosis is an important feature of any morphogenetic process, be it development or regeneration (Bastida *et al.*, 2004; Hwang *et al.*, 2004; Mallat *et al.*, 2005; Tseng *et al.*, 2007). Apoptosis is not only required to clear the damaged cells but this regulated cell death may also be essential for balancing the process of extensive cellular proliferation and useful for tissue patterning, which are characteristics of any morphogenetic event. In fact, an endogenous early apoptotic event is reported to be required for regeneration despite the massive tissue proliferation involved (Tseng *et al.*, 2007). Reports from different studies suggest that apoptosis inhibition in the early phases post amputation not only abolished regeneration but also affected proliferation and neuronal patterning of *Xenopus* tail regeneration buds (Tseng *et al.*, 2007). Moreover, refractory stage tail amputation resulted in an increase in the apoptotic population in the bud as compared to regenerative stages, suggesting that a tight control of a specific level of apoptosis is required for proper regeneration (Tseng et al., 2007). Whether BMP signaling mediates control of such an event during *Poecilia latipinna* tail regeneration is not known. Since BMP signaling inhibition delayed the regenerative event, its role if any, in regulating the apoptotic event during teleost epimorphosis was evaluated. From the results obtained from Caspase-3 localization in tail regenerates of control and LDN193189 treated fishes, it could be observed that BMP signal inhibition leads to an alteration in apoptosis during regeneration. LDN193189 treated animal showed an elevated level of cell apoptosis during blastema stage of the experiment. Moreover, sites and intensity in the apoptosis also showed differences. The LDN193189 treated fishes did not follow the uniformity in a pattern of apoptosis. Higher levels of apoptosis at all these sites were observed in the treated group. It is possible that BMP signal inhibition might have led to this increased cell death, since like TGF-β, BMP stimulates changes in the transcription of genes that regulate cell death and greater apoptotic activity in this group could be attributed to the delayed regeneration that in turn might have delayed the morphogenesis causing poor histoarchitecture of the fin. Hence, we conclude that apoptosis during the first 60 hr of regeneration is necessary for regeneration. Taken together, these findings indicate that BMP signaling has an important role during blastema stage of fin regeneration in *Poecilia latipinna*.

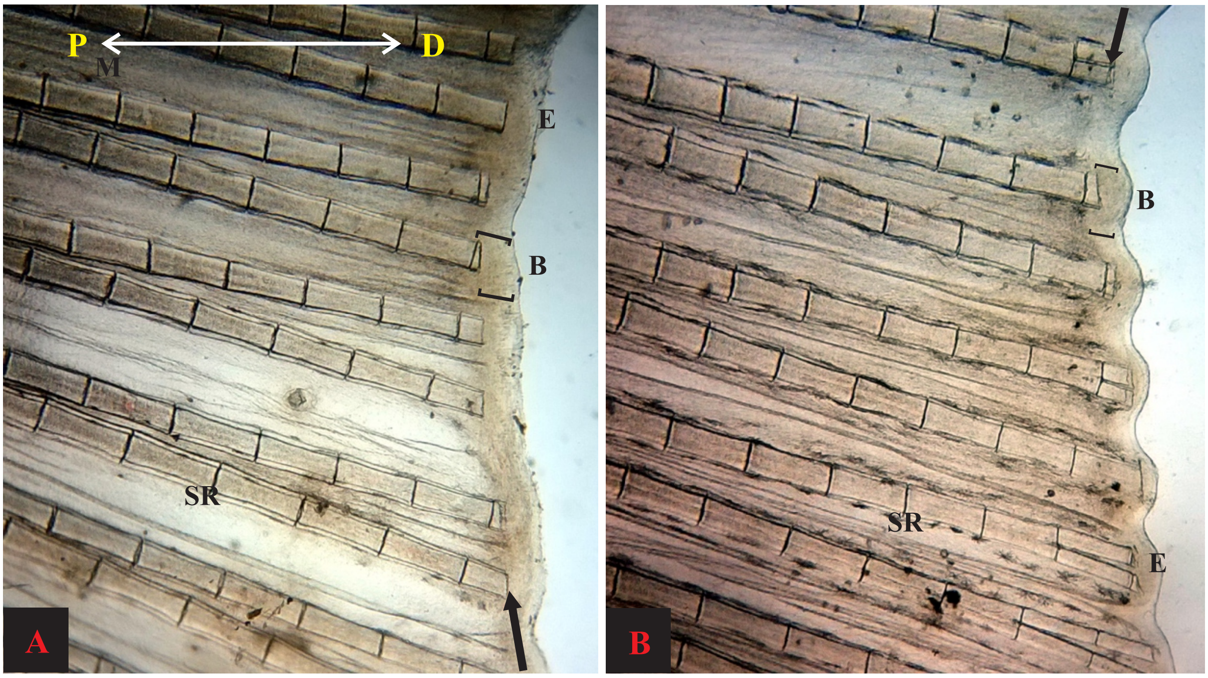

Figure 2.1: Morphological structure of caudal fin at 60hpa. Fin regeneration is subdued by LDN193189 treatment (B) when compared to DMSO control (A). The unusual pattern in the regenerated tissue can be clearly observed in the fin of LDN193189 treated animal.

E: Epidermis, B: Blastema (indicated by a square bracket for a single ray), SR: Segmented Ray, P: Proximal Direction, D: Distal direction. Black arrows indicate amputation plane; Magnification 4X

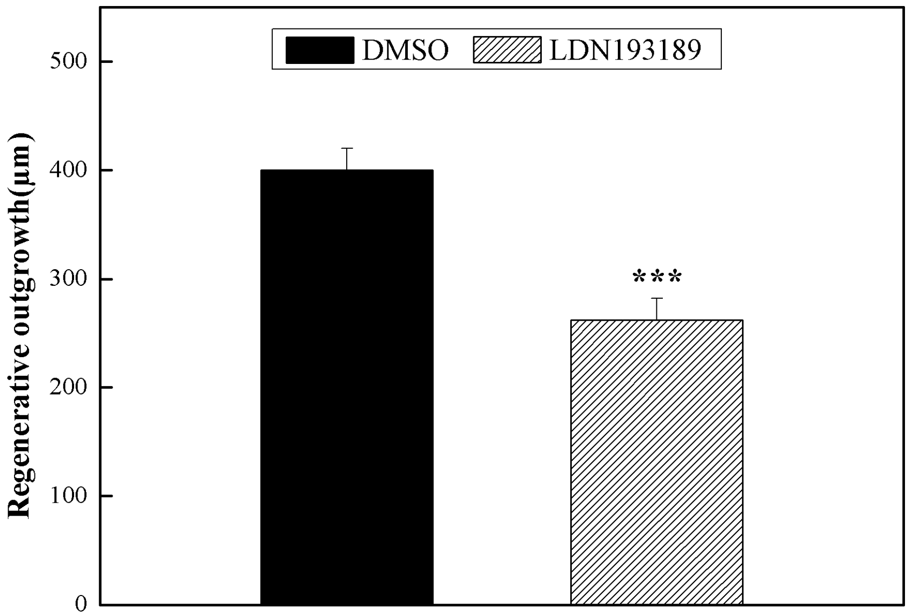

Figure 2.2: Morphometric analysis of caudal fin regenerate at 60hpa. Fish were treated with DMSO or LDN193189.Mean length of fin regenerates (n=8); ***P<0.001

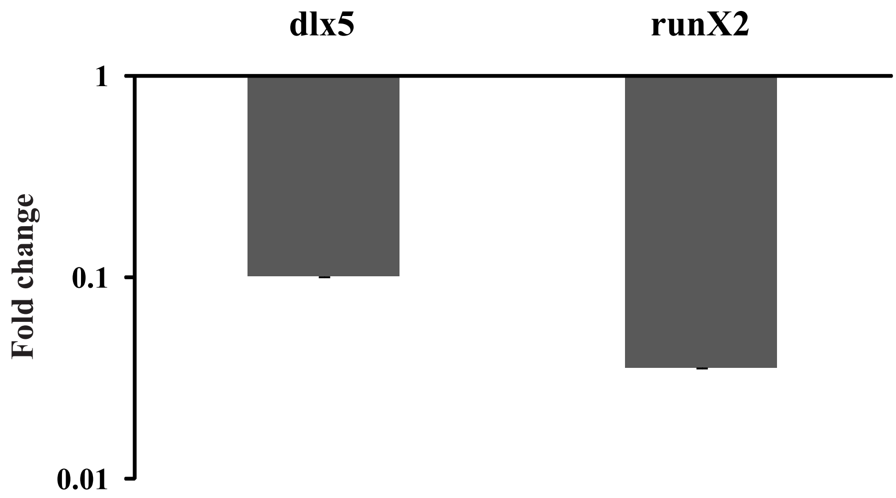

Figure 2.3: Mean fold change ($2^{-\Delta\Delta Cq}$) in the gene expression of dlx5 and runX2 in LDN193189-treated animals as compared to control at 60hpa. Error bars represent standard error of mean; n = 8

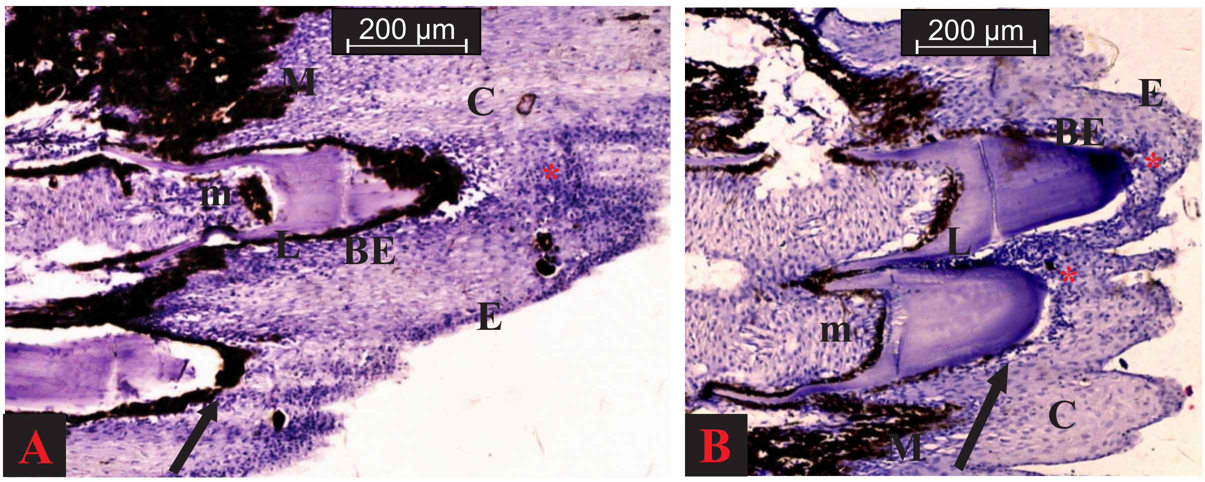

Figure 2.4 Histology profiles :Longitudinal Section of the distal region of the caudal fin of fish at 60hpa of regeneration from DMSO control and LDN193189 treated group. Black arrows indicate amputation plane. Blastema is marked by red asterisk (*)

E: Epidermis, m: Mesenchyme, M: Melanocyte, BE: Basal Epidermis layer, C: Connective tissue, L: Lepidotrichia.

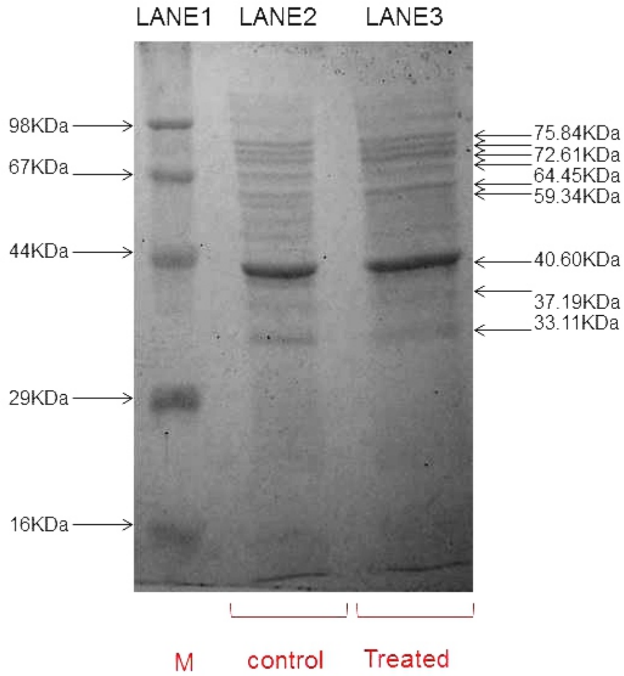

Figure 2.5 PAGE image of protein extracts from caudal fins of control and LDN-treated fish at 60hpa. Peptides showing significant difference in expression between control and treated samples have been labeled with their molecular weights.

Molecular weight (approx in kDa)	Relative band intensity (in arbitrary units)	
	Control	Treated
75.84	6.49	14.52
72.61	5.98	16.60
64.45	3.41	15.28
59.34	8.96	0
40.60	57.37	57.72
37.16	4.52	2.97
33.11	14.52	11.57

Table 2.1: Relative band intensities of peptides in control and LDN-treated group fins at 60hpa.

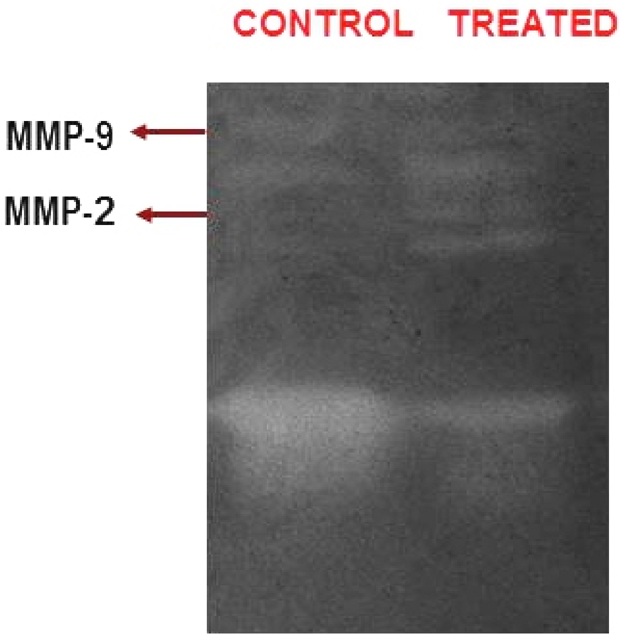

Figure 2.6 Zymogram showing activities of gelatinases MMP-9 and MMP-2 as clear bands against dark stained background at 60hpa.

Gelatinase	Relative band intensity (in arbitrary units)	
	Control	Treated
Pro-MMP-9	68.67	73.1
MMP-9	66.72	97.35
Pro-MMP-2	48.06	60.26
MMP-2	50.12	63.16

Table 2. 2: Relative activities of gelatinases (in terms of band intensities) in control and LDN-treated group fins at 60hpa

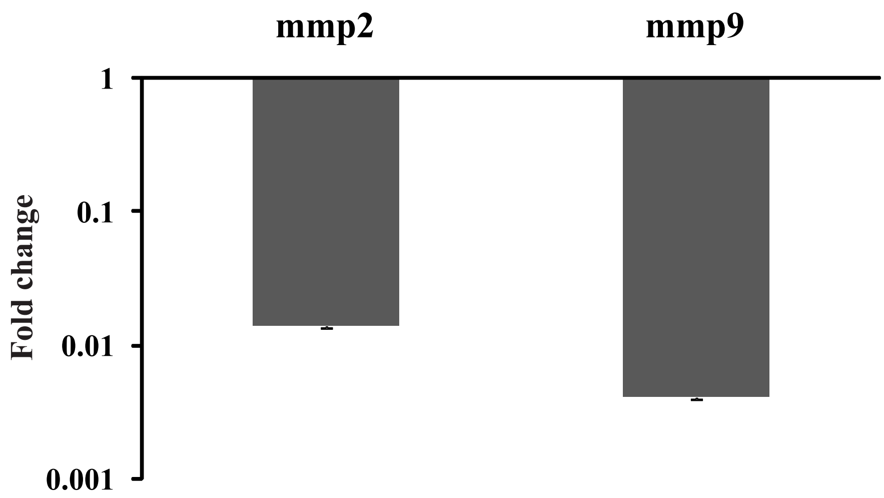

Figure 2.7: Mean fold change ($2^{-\Delta\Delta Cq}$) in the gene expression of mmp2 and mmp9 in LDN193189-treated animals as compared to control at 60hpa. Error bars represent standard error of mean; n = 8

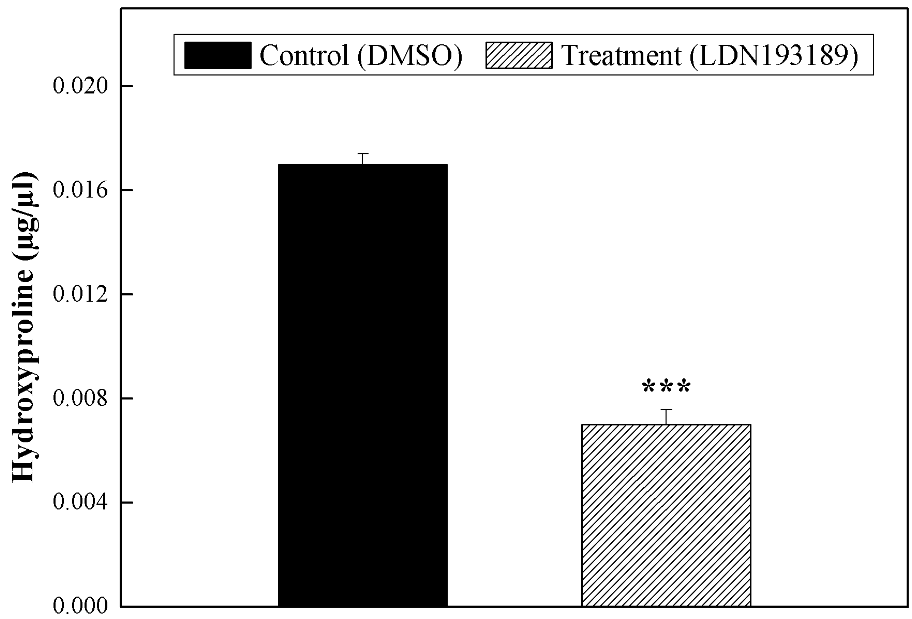

Figure 2.8: The collagen content of the fin tissue at 60hpa was assessed by a hydroxyproline assay.The collagen production of fin tissues was significantly decreased (***P < 0.001) at 60hpa in the fins of LDN193189 treated group animals in relation to fins of the DMSO control group animals.

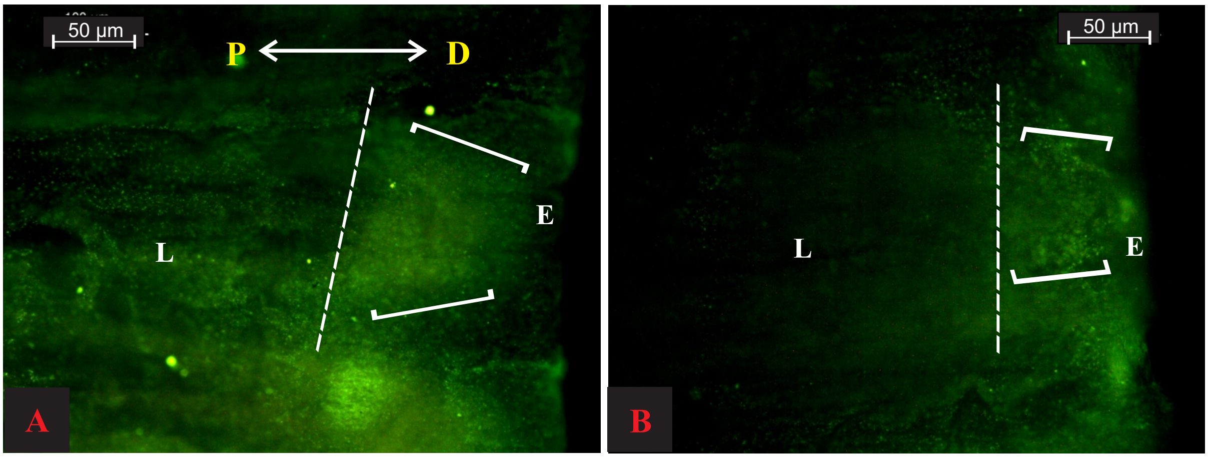

Figure 2.9: Immunofluorescence showing BrdU labeling in regenerating caudal fin at 60hpa . BrdU positive proliferating cells are green. BrdU-dense blastemal mesenchyme (A) and comparitively less dense in LDN193189 treated animal fin (B).Fins also showed non-specific epidermal fluorescence at the distal edge of the regenerate (previously reported by Poss *et al*., 2002b).Square brackets indicate blastema of a single ray. The dotted line indicates the approximate amputation plane.

L: Lepidotrichia, E: Epidermis, P: Proximal , D: Distal direction

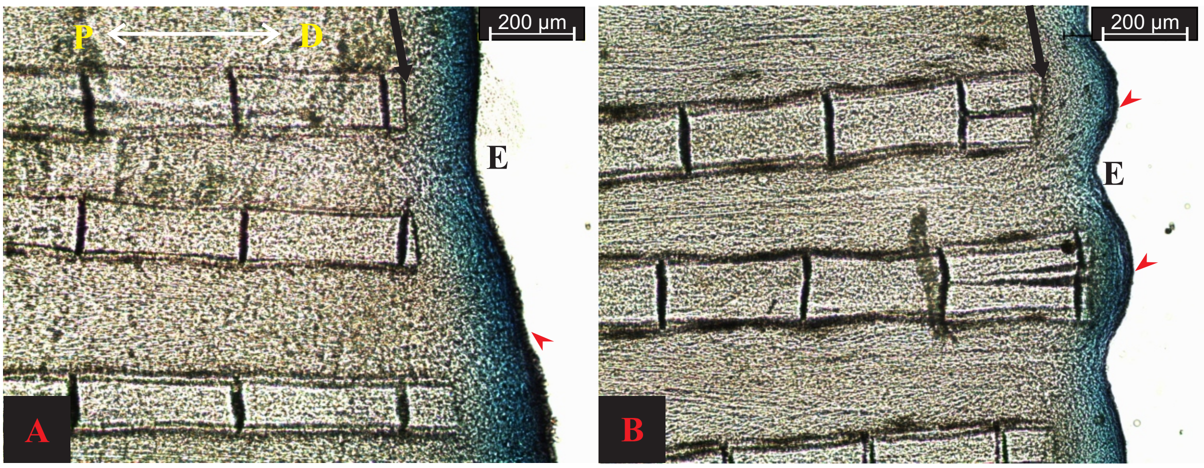

Figure 2.10: Immunohistochemical detection of activated caspase-3 at 60hpa during fin regeneration. Control fin (A) LDN193189 treated fin (B). Black arrows indicate amputation plane. Red arrowheads indicate areas of apoptosis.

E: Epidermis, P: Proximal, D: Distal direction