CHAPTER 1

INHIBITION OF BMP SIGNALING REDUCES MMP-2 AND MMP-9 EXPRESSION AND OBSTRUCTS WOUND HEALING IN REGENERATING FIN OF TELEOST FISH *POECILIA LATIPINNA*.

INTRODUCTION

An insult to any body part or tissue is a disruption of its morphology and/ or functionality. However, after injury, structural tissue heals with incomplete (Velnar *et al*., 2009) or complete regeneration (Coletti *et al*., 2013). Besides, a tissue without any measurable disruption to morphology almost always completely regenerates. The best example which can be cited here is non-injured skin, which exhibits constant replacement and regeneration of cells resulting in complete regeneration (Alberts *et al*., 2008).

Wound healing is the process by which body tissue repairs itself after trauma. The process is not only complex but also fragile, and it is susceptible to interruption or failure which may lead to the formation of non-healing wounds. In uninjured skin, the epidermis or surface layer and dermis or deeper layer form a defensive barrier against the external environment. When this barrier is broken, an orchestrated cascade of biochemical events is set into motion to repair the damage (Rieger *et al*., 2014).

Wound healing chiefly comprises inflammation, apoptosis of damaged cells, extracellular matrix (ECM) rebuilding by proteases leading to cellular migration for covering the wound surface and ultimately cellular proliferation to heal the wound (Rieger *et al*., 2014). Soon after amputation, the epidermis covers the wound to form an epidermal cap. Epidermis is a stratified cellular sheet and all the outside-in and inside-out barrier functions are dependent on it. It has been documented that epidermis plays an important role during regeneration in various groups of animals (Poleo *et al.*, 2001). When all these multivariate events are happening, concurrently re-epithelialization of the epidermis occurs, in which epithelial cells proliferate and crawl over the wound bed, providing cover for the new tissue (Garg, 2000).

During epimorphic regeneration, immediately after amputation, epidermal cells from the circumference of the lost part migrate to cover the exposed mesenchymal tissues (Bryant *et* *al*., 2002). This wound healing phase is achieved strictly through cell movement without cell division (Hay and Fischman, 1961). Subsequent blastema formation and repatterning cannot occur unless the amputation wound has healed, hence it has been largely established that the wound healing phase plays an important role in the initiation of the regenerative process (Campbell and Crews, 2008).

Early during regeneration, epidermis at the tip lacks a complete basement membrane (Young *et al.*, 1985) and this state is supposed to be critical for early epithelial-mesenchymal signaling events involved in regeneration (Stocum, 1995a). Also, relatively low and brief inflammatory response favors tissue regeneration (Wynn, 2008). In contrast, intense inflammatory reaction and the presence of a granulomatous reaction do not allow the establishment of a mesenchymal population, and the rapid formation of a continuous dense lamella in the basement membrane blocks any dermal-epidermal interactions to sustain the elongation of the lost part. Ferguson and O'Kane (2004) and Alibardi (2010) have reported that all these combined factors are characteristic of wound healing with scar formation in organisms after an injury.

The process of wound healing involves a series of rapid increases in specific cell populations that prepare the wound for repair, deposit new matrices and finally, mature the wound. During maturation and remodeling, collagen is realigned along tension lines, and cells that are no longer needed are removed from the wound prior to the progression to the next phase of healing. The most logical method of cellular down-regulation is through programmed cell death (PCD) or apoptosis. Apoptosis allows for the eliminations of entire populations without tissue damage or an inflammatory response. Apoptosis expectedly plays an important role in determining the regenerative ability (Elmore, 2007). Moreover, studies on a cellular aspect of *Xenopus* tail regeneration by Tseng *et al.,* (2007) revealed that apoptosis is required during the first 24hpa. When Caspase-3 activity is inhibited, regeneration is abolished. Tseng *et al.,* (2007) interpreted these results to mean that there may exist endogenous inhibitory cells that must be destroyed by programmed cell death for regeneration to occur. Further, cell-cell and cell-matrix interactions during matrix reorganization are essential in healing and apoptosis might be involved in regulating these interactions.

Different events of wound healing require the vigorous action of various proteolytic enzymes. Indeed, tissue breakdown, cell movement, cell-cell and cell-matrix interactions and dedifferentiation can occur only if major alterations in the extracellular matrix take place (Tsonis *et al.*, 1996). Extracellular matrix plays a dynamic role during the process of wound healing, embryogenesis, and tissue regeneration. Recent studies have suggested that in addition to providing structural stability, the ECM also acts to sequester and store growth factors, direct them to their receptors and sense and transduce mechanical signals (Kim *et al.*, 2011; Hynes, 2014). Therefore, it could be deduced that the biochemical and the mechanical cues provided by the ECM play critical roles in regulating cell behaviors including migration, shape, survival, differentiation, and proliferation. Moreover, remodeling of the ECM occurs during normal development, morphogenesis, wound healing and in diseased states such as cancer (Daley and Yamada, 2013; Tolg *et al.*, 2014). Also, ECM remodeling has recently been shown to contribute to epimorphic regeneration of newt skeletal muscle and heart, *Xenopus* tadpole tails, and zebrafish heart (Toole and Gross, 1971; Gulati *et al*., 1983; Tassava *et al*., 1996; Calve *et al.*, 2010; Mercer *et al*., 2013)

Timely degradation of ECM is an important feature of development, morphogenesis, tissue repair and remodeling which is precisely regulated under normal physiological conditions (Nagase *et al*., 2006). Various types of proteinases are implicated in ECM degradation, but the major enzymes considered to be primarily responsible for ECM turnover are the matrix metalloproteinases (MMPs), also called matrixins (Visse and Nagase, 2003). These are involved in digestion of the extracellular matrix, which helps cell migration, proliferation and also cell-to-cell communication. Members of the MMP family have been implicated in epithelial wound healing of the cornea, the skin (Azar *et al*., 1996; Fini *et al*., 1998; Ye and Azar, 1998), in the regeneration of tissues including mouse muscle (Kherif *et al*., 1999), zebrafish heart and fin (Bai *et al*., 2005; Lien *et al.*, 2006), and urodele appendages (Yang and Bryant, 1994; Miyazaki *et al.*, 1996; Yang *et al.*, 1999; Vinarsky *et al.*, 2005). MMPs are mainly produced by cells of mesenchymal and hematopoietic lineages (Birkedal-Hansen *et al*., 1993) and certain MMPs are expressed in specific embryonic and extra-embryonic tissues. Neutrophils (Schwartz *et a*l., 1998) and macrophages infiltrating through vessel walls and vascular basement membrane are key producers of MMPs in wound healing (Yong *et al.*, 1998). These cells have the potential to cleave and degrade type IV collagen in the basement membrane and collagens type I and III in the ECM. MMPs are not expressed, however, by stationary tissue fibroblasts (Hakkinen *et al.*, 2000). MMPs have been known to show an important role in the breakdown of the conditional matrix formed after wounding by breaking down several components of the ECM, including type IV collagen, fibronectin, laminin, entactin and elastin, as well as clarifying the cellular and fibrillar debris that surround the wound bed (Baker and Leaper, 2000).

Further, it was observed that during wound healing, TGF-β stimulates fibroblasts to migrate into the injured area, where they proliferate and bring about the contraction of the wound margins enabling wound closure (Werner and Grose, 2003). Along with TGF-βs and activins, BMPs have also been suggested to play a role in wound repair (Werner and Grose 2003). BMP-2, BMP-4, and BMP-7 are expressed in normal and wounded adult mouse skin, although their expression is not regulated by skin injury (Wankell *et al.*, 2001). The sites of expression of these proteins in the wounded skin and their roles in wound repair have not yet been determined, but exogenous BMP-2 has been found to induce massive dermal and epidermal growth in fetal wounds of lambs and an adultlike pattern of scar formation (Stelnicki *et al.*, 1998). Despite many years of study, very few studies have been reported showing the roles of various signaling molecules at wound epithelium stage. Several endogenous antagonists of the BMP signaling pathway have been recognized, including Noggin, which prevents BMPs from binding to the transmembrane receptors (Ben-Arie *et al.*, 1997). If a noggin expression is induced followed by partial amputation of the tail i*n Xenopus*, then wound closure and the formation of the neural ampulla occur normally but an outgrowth of the regeneration bud is inhibited. BMP signaling is also involved in AEC migration and is likely to mediate the switch towards an EMT-like phenotype by altering protein expression to facilitate cell migration and wound closure (McCormack *et al.*, 2013). The same report also suggested the involvement of BMP antagonists noggin and gremlin during inhibition of cell migration, confirming the involvement of BMP signaling in migration and indicating autocrine signaling, possibly involving BMP-7 or BMP-4 which were expressed in AECs. In contrast to other BMPs, the expression of BMP-6 in healing skin wounds has been well documented. It is highly expressed in the regenerating epidermis at the wound edge as well as in fibroblasts of the granulation tissue. After completion of wound closure, BMP-6 was found to accumulate throughout the suprabasal layers of the newly formed epidermis (Kaiser *et al.*, 1998)

Teleost fishes have been extensively used as a model to study tissue regeneration, since they possess the widespread capability of partly or wholly restoring damaged spinal cord (Becker *et al.*, 2004; Bareyre 2008; Reimer *et al.*, 2008; Goldshmit *et al.*, 2012), heart (Poss *et al.*, 2002; Raya *et al.*, 2004; Schnabel *et al.*, 2011; Yin *et al.*, 2012), retina (Qin *et al.*, 2011) and fins (Johnson and Weston, 1995; Lee *et al.*, 2010; Azevedo *et al.*, 2011; Blum and

Begemann, 2012; Singh *et al*., 2012). Caudal fin regeneration in the teleost fish represents an excellent model to understand epimorphic regeneration (Poss *et al*., 2003).

Besides their role in skeletal development, BMPs also regulate a wide range of cellular processes including proliferation, differentiation, motility, adhesion, cell death and specification of developmental cell fate during embryogenesis (Hogan, 1996b; Massague´, 2000; Wozney, 2002; Yamamoto and Oelgeschläger, 2004). Moreover, a variety of model systems both *in vitro* and *in vivo* have shown the importance of TGF-β members in these processes (Langenfeld and Langenfeld, 2004; Satoh *et al*., 2005; Boyd *et al*., 2007).

The present study was undertaken to understand the deviation, if any, in the multivariate interactions that normally occur near the site of amputation involving the immune system, apoptosis and extracellular matrix after BMP signaling inhibition. This may aid in designing tissue engineering and wound healing strategies that directly control the healing response in a way favourable to regeneration. LDN193189, a potent inhibitor of BMP signaling, which functions through blockage of BMP type 1 receptors (Cuny *et al*., 2008), was used in this study to inhibit the BMP signaling.

MATERIAL 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. The animals were maintained under standard conditions of humidity, temperature and light/dark photoperiod (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

Animals were acclimatized for a week before beginning the study. They were divided into two groups of 24 each: The control group received injections of 1% dimethyl sulfoxide (DMSO), while treatment group received injections of LDN193189 (prepared freshly in 1% DMSO) at 2.5mg/kg body weight. The drug was administered intraperitoneally, using a microsyringe (25µL, Hamilton Bonaduz AG, Switzerland). One-third of the original caudal fin from distal to proximal direction was amputated using disposable sterile stainless steel surgical blade, and after few minutes each group was treated and the fishes were released immediately into the tanks with full care.

Morphometric study

The time taken to achieve the wound epithelium 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.

Histology

Tissues collected for histology were preserved in 10% formalin. These were processed for paraffin embedding. 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.

SDS-PAGE

Regenerating tail fin tissue from each experimental group was collected 24hour post-amputation (hpa), pooled (n=08) 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

7.5% SDS-polyacrylamide gels were used with gelatin (5mg/ml). After electrophoresis, gels were washed with 2.5% Triton X-100, followed by a wash in an incubation buffer and then 18hr incubation with the same at 37^{o}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.

Western Blot

Tissue samples were processed as mentioned for SDS-PAGE. A total of 25µg protein was electrophoresed on a 10% SDS-PAGE gel. The proteins were electrophoretically transferred onto a nitrocellulose membrane (0.45µm pore size) at 30V overnight. The transfer was confirmed by reversible staining using Ponceau. The membrane was probed for MMP-2 and

MMP-9 using IgG antibodies raised in rabbit (at 1:1000 dilution). Biotinylated goat anti-rabbit IgG was used as secondary antibody for detection of proteins. Band was developed using an ALP-based staining method. The internal control used here was beta-actin. Band intensities were measured and compared by densitometric analysis using Doc-ItLs software (GeNei, Merck, USA).

Real-time RT-PCR

Total RNA was isolated from regenerating fish fin homogenate 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
18srRNA	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGGCTGAAC

Quantitative real-time PCR was performed on a LightCycler 96 (Roche Diagnostics, Switzerland) with the following program: 3 min at 95^{o}C, 45 cycles (each cycle of 10s at 95^{o}C, 10s at 60^{o}C and 10s at 72^{o}C). 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).

Immunohistochemistry of Caspase-3

As Caspase-3 is the main executioner of apoptosis, IHC of the active form of Caspase-3 (active casp-3) was performed to check the apoptosis in the fin whole mounts. For this zone of interest was collected on 24hpa and processed as per the procedure described earlier in detail.

Statistical Analysis

All linear data obtained are expressed as a mean ± standard error. Statistical significance between two means was computed in SPSS program using unpaired Student’s t-tests. A p-value less than or equal to 0.05 was considered statistically significant.

RESULTS

Validation of BMP signaling inhibition by LDN193189

We first confirmed whether the intraperitoneal administration of LDN193189 (LDN) successfully hampered BMP signaling in the fish fin. 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 RT-PCR. These genes dlx5 and runx2 were found to have a significantly low expression in the treated fins as compared to controls (Figure 1.1), with a fold change of 0.0028 and 0.00012, respectively, thus proving the inhibition of BMP signaling in the test organism treated with the selected dose of LDN193189.

LDN193189 treatment causes incomplete covering of amputation wound

As a normal process following fin amputation in *Poecilia latipinna*, the epithelial cells cover the amputation site by 24hpa. This process, completing into what is called wound epithelium stage, can be observed as smoothening of the fin at the cut edge. Epithelial cells migrate to cover the distal end and subsequently form a thick layer of cells. This actively secreting structure provides the environment favorable for recruitment of pluripotent cells underneath, which will eventually form the blastema. Morphological observations revealed the presence of a thin wound epithelium on the distal margins of the regenerating tail in LDN193189 treated animals depicting stunted progression of the re-epithelialization as compared to that of control group animals wherein a thick wound epithelium was evident by 24hpa (Figure 1.2). Moreover, LDN193189 treated animals showed reduced regenerative outgrowth in a manner that was significant as compared to that of controls ($p \leq 0.05$) (Figure 1.3). Also observed that the outer covering of epithelium at the amputation surface of the fin in the treated group of fishes was not as smooth as that of control group fishes on 24hpa (Figure 1.2). This observation, however, needed to be supplemented with histological analysis for clarity of events occurring at the cellular level. Histological profile of the tail showed that at the wound healing stage, a smooth covering of cells was seen over the distal end of each lepidotrichium (bony ray supporting fin structure). Also appearing at the distal end were melanocytes and other cells of the mesenchyme. While these features were distinctly visible in the tail fin sections of control group animals (Figure 1.4A), they were inconspicuous in the treatment group fins (Figure 1.4B). Treatment group fins were lacking in a properly covered amputation wound, as can be understood from the absence of epithelial cells, conjunctive tissue, and pigment cells.

BMP signaling inhibition altered epidermal apoptosis

It is evident from the Caspase-3 enzyme activity in the immunohistochemically stained tail fins that at 24hpa intense programmed cell death was limited closer to the site of amputation in the LDN193189 treated fishes (Figure 1.5B). Contrastingly, the fins from the control group showed the uniformly high activity of active Caspase-3 on the entire surface of the fin which, however, showed a declining trend towards the wound epithelium (Figure 1.5A).

Protein profile of wound epithelium is altered due to LDN193189 treatment

The healing process leading to regeneration involves a major change in the expression pattern of peptides from the resting stage. The induction of new peptides having varied roles in the regenerative process occurs shortly following amputation (Poss *et al*., 2003). Whether the inhibition of BMP signaling affects the expression pattern of peptides during the initial stages of regeneration or not in the selected test system was studied using SDS-PAGE. Analysis of the gel image revealed that the intensity of a large number of bands decreased following LDN193189 treatment. The bands with the most significant reduction in intensity were at approximately 93, 83, 70, 62, 48, 40 and 32 kDa (Figure 1.6; Table 1.1).

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

The gelatinases, MMP-2 and MMP-9, are responsible for one of the most fundamental and important processes in regeneration. These enzymes digest parts of the extracellular matrix to allow mobilization and proliferation of cells throughout the process of growth (Yang *et al*., 1999). Since these enzymes are thought to contribute largely during the very initial stages of growth and repair, a zymographic analysis was carried out at 24hpa to study the effect of BMP inhibition on their activities. Results from the analysis of zymogram reflected a sharp decline in the activities of the gelatinases in the regenerating tail in response to LDN193189 treatment (Figure 1.7). Intensities of bands corresponding to pro-MMP-9 as well as MMP-9 were found reduced from 100 and 103.85 (in arbitrary units) to 69.63 and 47.03, respectively, to treatment with the test substance. Furthermore, pro-MMP-2 activity decreased from 69.93 to 38.65 and MMP-2 activity dropped from 30.51 to 25.21 after treatment with the BMP inhibitor (Table 1.2).

Immunoblot analysis of MMP-2 and MMP-9 peptides in the regenerating tail fins further consolidated the above observation with a definite decrease in the expression of both the peptides in the tail tissues of LDN193189 treated fish as evident from the relatively low intensity of bands in the blot compared to that of control (Figure 1.8; Table 1.3).

MMP-2 and MMP-9 gene expression by real-time RT-PCR

In order to reaffirm the influence of BMP signaling in the regulation of gelatinases during tail regeneration in fish, further transcript level analysis was carried out. The results of the real-time RT-PCR showed a distinct decrease in the expression of both mmp2 and mmp9 genes in the samples collected from LDN193189 treated fish (Figure 1.9). The mean fold decrease for mmp2 and mmp9 expression was 0.038 and 0.019, respectively, as compared to the controls.

DISCUSSION

Upon amputation, the restoration process is initiated by a wound healing phase which is the first major event that decides whether regeneration will or will not take place. The healing process is accompanied by a plethora of changes at the levels of cell structure, function and gene expression. A fraction of cells at the plane of amputation undergo programmed cell death. Programmed cell death, is intimately involved in the regulation of inflammation and ultimately should play a role in the inflammatory phase of wound healing. To check this notion immunohistochemistry was performed on the fin whole mount to detect active Caspase-3 in 24hours post amputated fins of both groups during wound epithelium stage. BMP signaling inhibition had a noticeable effect on cell cycle or apoptosis. Proliferating cells were absent during the wound closure. Moreover, the epidermal cells at the wound site were non-proliferating cells.

After undergoing PCD cells near the amputation plane gave way to a marching line of epithelial cells, which cover the wound completely around 24hpa in *Poecilia latipinna.*

Moreover, we did find a significant decrease in the expression of both these genes (dlx5 and runx2) in the healing caudal fin at 24hpa, confirming the inhibition of BMP signal (Chen *et al.,* 2012). Further analyses with a morphology and histology based experiment to check for any effect of LDN193189 on the migration of epithelial cells and normal covering of the wound revealed significantly retarded covering of the distal tip of the fins in treated animals. The covering and smoothening of the distal ends of lepidotrichia by migration of epithelial cells over their surface were not as notable in treated fins as in the control fins of the same stage. The known role of BMP members in the induction of cell migration in various embryonic and post-embryonic development systems (Fiedler *et al*., 2002; Inai *et al.,* 2008; Christiaen *et al.,* 2010; Chen *et al.,* 2014) is reflected in the current study as well. However, it remains to be ascertained in our model system whether the downstream effects of BMP signaling on cell migration are direct or not.

Nonetheless, as expected, a number of new peptides were synthesized during this phase, and many must even be downregulated. A spot densitometric scan of the PAGE gels revealed downregulation of multiple peptides, whose specific bands of interest were between around 92 and 62kDa.

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. This is because MMPs are very well-known mediators of tissue remodeling after injury and play a crucial role in the initiation as well as in the further progression of epimorphic regeneration in vertebrates (Sharma *et al*., 2011; Annasamudram, 2012; Pillai, 2012).

Wound healing is associated with tissue reorganization at the site of injury, and extensive remodeling of the extracellular matrix (ECM) allows this process. At the forefront of ECM remodeling lies the matrix metalloproteinase family of enzymes. MMPs are secreted as proenzymes, which are then activated by cleavage. On activation, MMPs facilitate cell migration and proliferation by digestion of the collagen matrix in the local tissue (Visse and Nagase, 2003; Wang *et al*., 2009). Two of the MMPs widely attributed with the function of ECM digestion during regenerative healing are MMP-2 and MMP-9.

In the current study, it was thought pertinent to check whether the impeding effect of LDN193189 on wound healing is mediated through any alteration in the activities of the MMPs. Zymographic analysis revealed here that a major reduction in the activities of both the MMPs took place in response to LDN treatment. Pro-MMP-2 and Pro-MMP-9 activities were also reduced, suggesting that the decrease in metalloproteinase activities was likely due to a downregulation of their expression.

To verify this result, a western blot for MMP-2 and MMP-9 was carried out, and indeed the results obtained showed decreased expression of both the MMPs in treatment group samples. This led us to believe that the reduced activities of the MMPs after LDN193189 treatment are due, at least in part, to their downregulation and not wholly due to any kind of catalytic inhibition. To further understand whether this downregulation was occurring at the transcriptional level or at a level of peptide synthesis, a real-time RT-PCR was performed.

Results from our semiquantitative analysis clearly established that the suppression of expression of MMPs was occurring at the transcriptional level.

Therefore, our study reveals that inhibition of BMP signaling leads to downregulation of expression of mmp2 and mmp9 genes in the wound healing of an amputated teleost fish fin. While it was earlier known that BMP-4 blocks the expression of MMP-3 and MMP-13 in stem cells (Otto *et al.,* 2007), the role of BMPs in regulating MMP-9 was also subsequently brought to light in breast cancer cells (Shon *et al.,* 2009).

However, Shon *et al.,* found BMP-4 to be negatively regulating MMP-9 expression, whereas the regenerating model system in the current study reveals a positive relation between BMP signaling and MMP-9 expression. We do find congruence of our results with a few earlier studies.

Gordon *et al.* (2009) used a pancreatic cancer system and showed that BMPs act through a Smad1 dependent pathway to increase the expression of mmp2 and induce cell invasiveness. TGF-β signaling has been shown to induce mmp2 expression in gingival fibroblasts (Overall *et al.,* 1991) and mmp9 expression in a number of cell types (Salo *et al.,* 1991; Tjäderhane *et al.,* 1998; Festuccia *et al.,* 2000).

These findings further consolidate our present notion of the inevitable involvement of BMP signaling in apoptosis and facilitating wound closure at the site of amputation by modulating the expression of mmp2 and mmp9 in teleost fish.

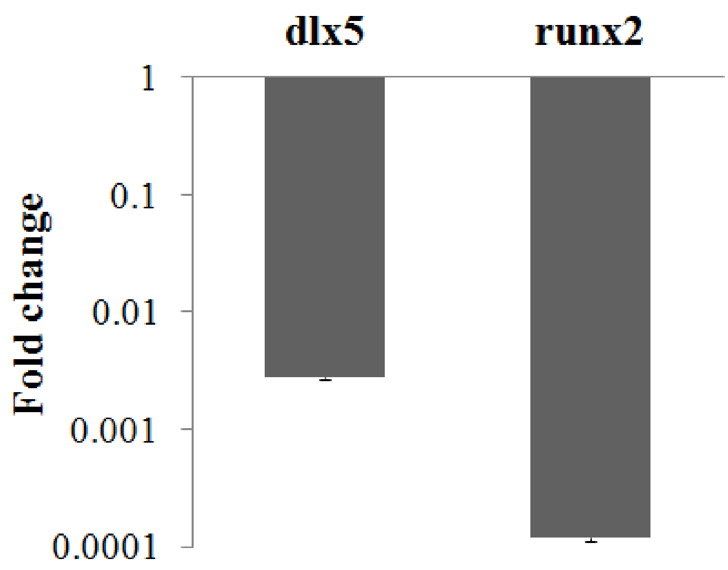

Figure 1.1: Mean fold change ($2^{-\Delta\Delta Cq}$) in the gene expression of dlx5 and runX2 in LDN193189-treated animals as compared to control. Error bars represent standard error of mean; n = 8

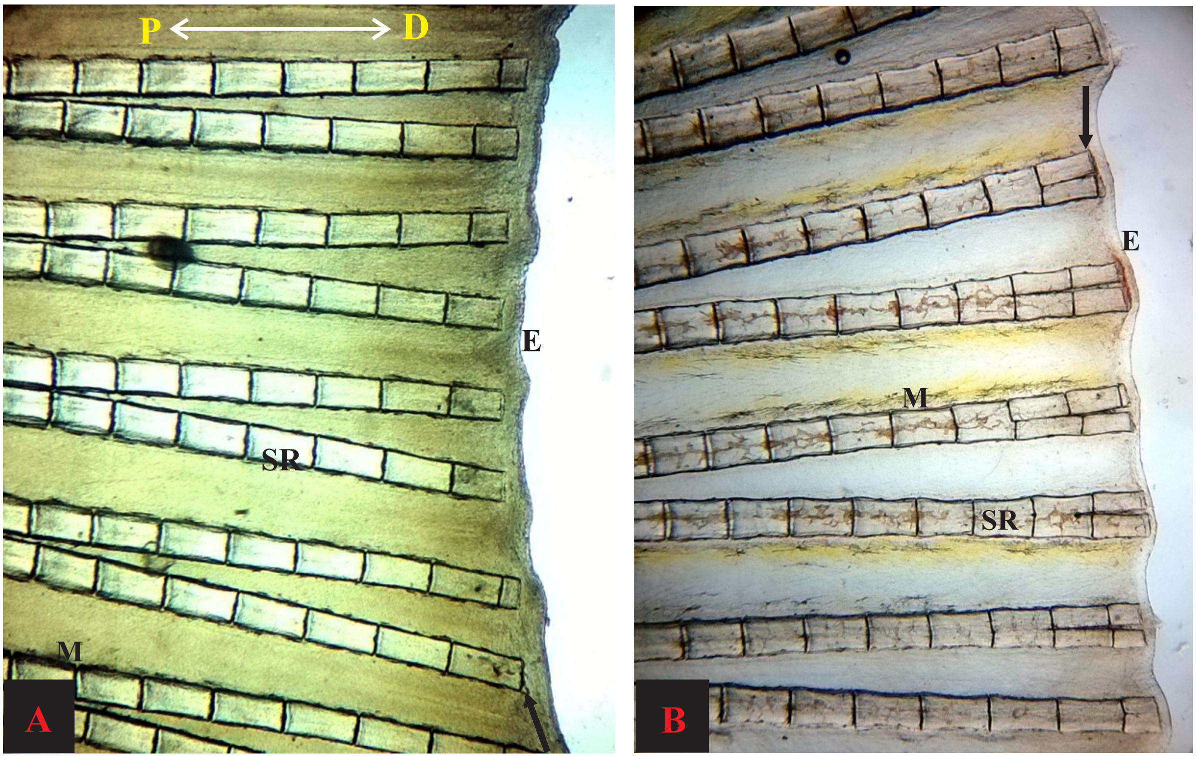

Figure 1.2: BMP signaling is required for the normally initiated regeneration. Bright-field images of DMSO control (A) and LDN193189 treated (B)fins of the animals at 24hpa at normal conditions. Black arrows indicate amputation plane.

E epidermis, SR segmented ray, M melanocytes, P proximal direction, D distal direction; Magnification 4X

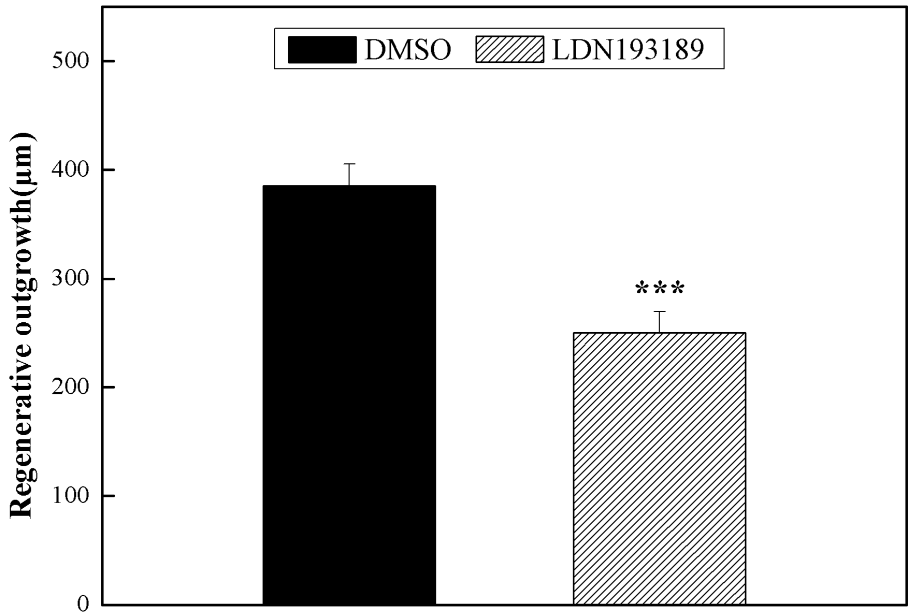

Figure 1.3: Morphometric analysis of caudal fin regenerate at 24hpa. Fish were treated with DMSO or LDN193189. Mean length of fin regenerates (n=8); ***P<0.001

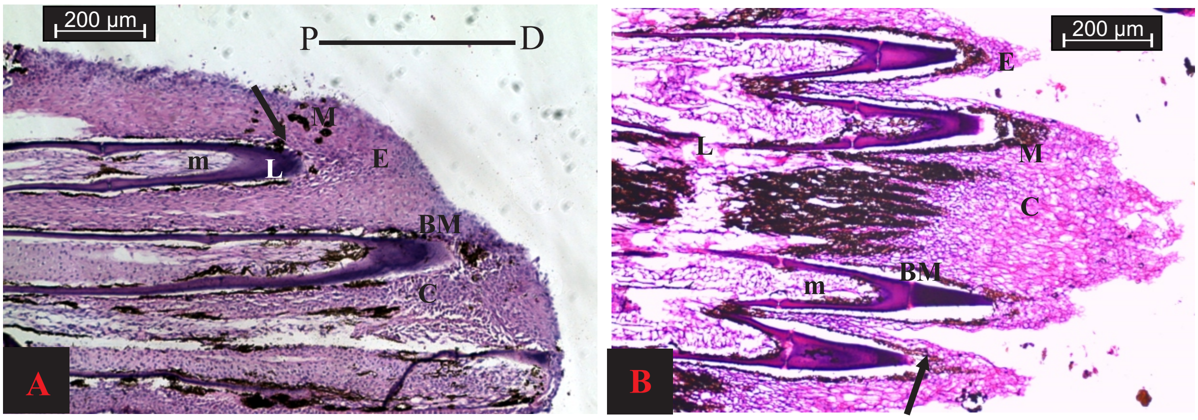

Figure 1.4: Caudal fin histology at 24 hpa in control and treated fish.
Thickened wound epidermis can be seen in control fins at 24 hpa (A) over the distal end of each lepidotrichium along with pigment cells. Treated fins show an incompletely covered wound epidermis (B). Black arrows indicate amputation plane.

E epidermis, BM basal membrane, C connective tissue, L lepidotrichia, m mesenchyme, M melanocytes, P proximal direction, D distal direction.

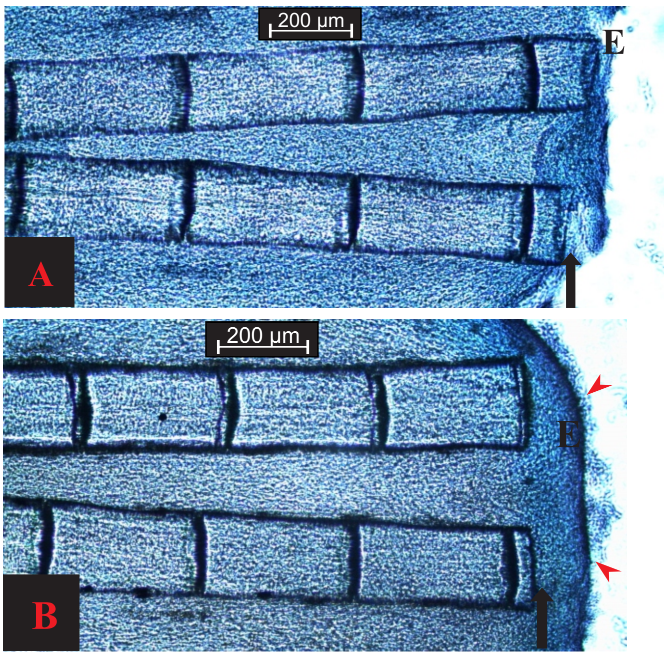

Figure 1.5: Immunohistochemical detection of activated caspase-3 at 24hpa during fin regeneration. Control fin (A) LDN193189 treated fin (B). Black arrows indicate amputation plane. Red arrowheads indicate areas of apoptosis.

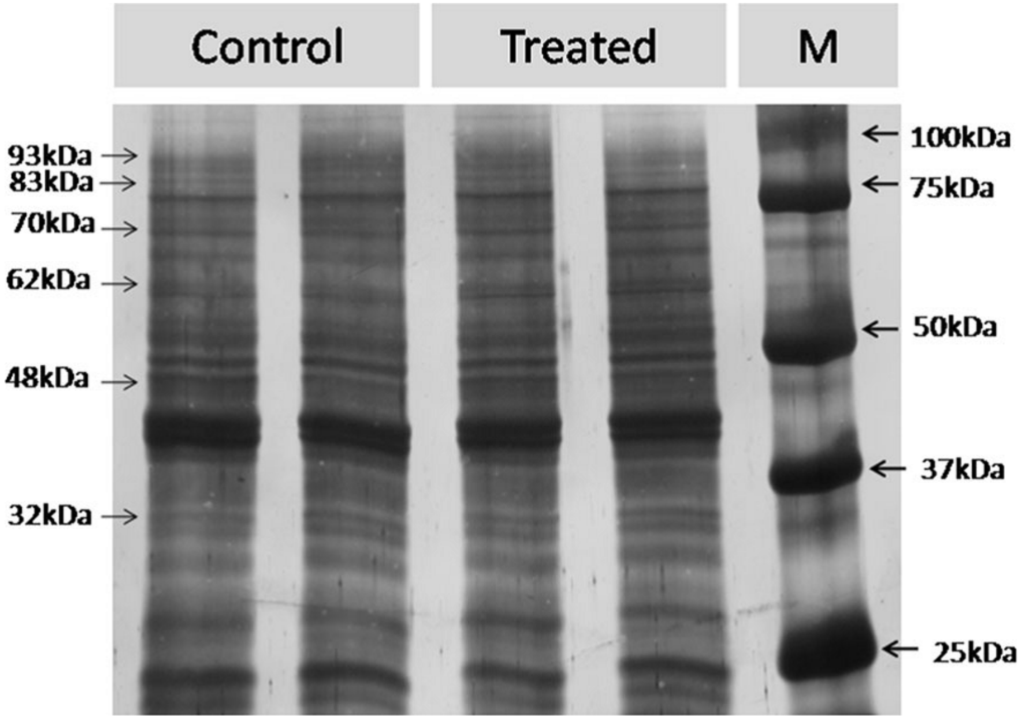

Figure 1.6: PAGE picture of protein extracts from caudal fins of control and LDN-treated fish at 24 hpa. Peptides showing significant difference in expression between control and treated samples have been labeled with their molecular weights

Molecular weight (approx in kDa)	Relative band intensity (in arbitrary units)	
	Control	Treated
93	54.30	44.56
83	61.86	38.69
70	46.12	31.23
62	49.15	28.94
48	89.93	61.11
32	68.16	43.54

Table 1.1: Relative band intensities of peptides in control and LDN-treated group fins at 24 hpa.

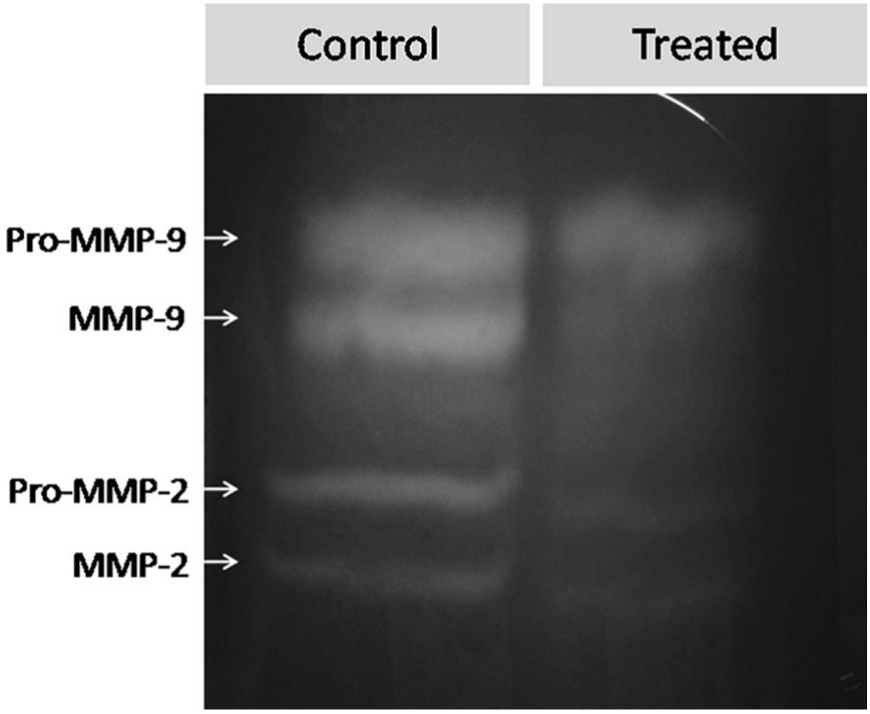

Figure 1.7: Zymogram showing activities of gelatinases MMP-9 and MMP-2 as clear bands against dark stained background

Gelatinase	Relative band intensity (in arbitrary units)	
	Control	Treated
Pro-MMP-9	100	69.63
MMP-9	103.85	47.03
Pro-MMP-2	69.93	38.65
MMP-2	30.51	25.21

Table 1.2: Relative activities of gelatinases (in terms of band intensities) in control and LDN-treated group fins at 24 hpa

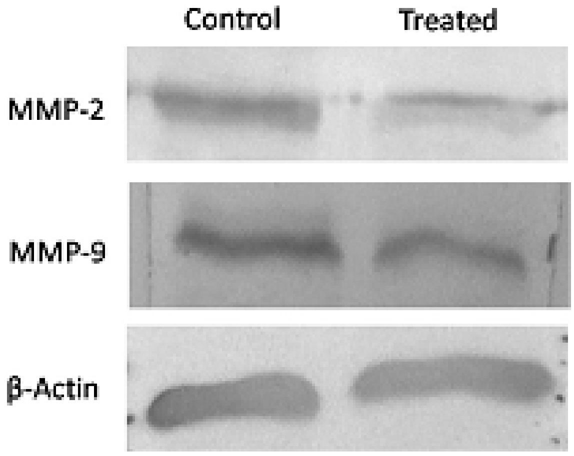

Figure 1.8: Western blot analysis of MMP-9 and MMP-2 in caudal fins of control and LDN-treated fish at 24 hpa

	Relative normalized band intensity (in arbitrary units)	
	Control	Treated
MMP-2	98.72	67.77
MMP-9	149.26	130.84

Table 1.3: Relative band intensities of MMP-2 and MMP-9 in control and LDN-treated group fins at 24 hpa, normalized against intensity of β-actin

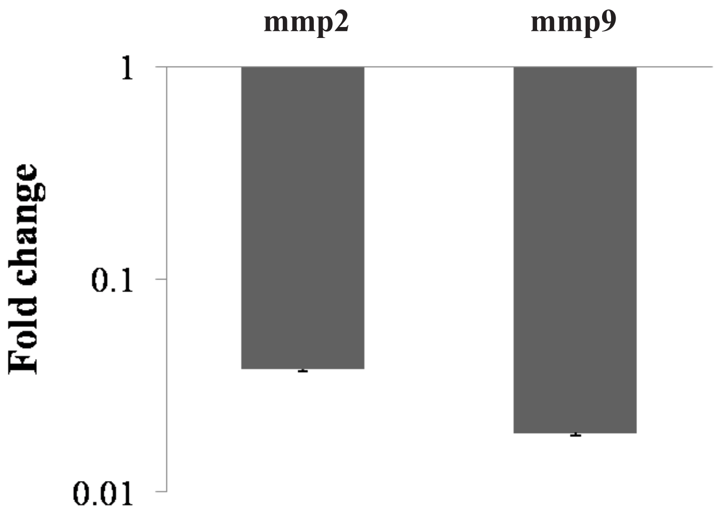

Figure 1.9: Mean fold change ($2^{-\Delta\Delta Cq}$) in expression of MMP-2 and MMP-9 in LDN-treated fins at 24 hpa as compared to control. Error bars represent standard error of mean; n = 8