CHAPTER 3

BMP SIGNALING REGULATES THE SKELETAL AND CONNECTIVE TISSUE DIFFERENTIATION DURING CAUDAL FIN REGENERATION IN TELEOST FISH.

INTRODUCTION

Epimorphic regeneration in teleost fin occurs through the establishment of a balanced growth state, in which a blastema gives rise to all the mesenchymal cells, the cells which differentiate and thus allowing the enlargement of the whole structure. In order to form a complex tissue structure, cells undergo differentiation by which mesenchymal cells develop into specific cell types by taking on shapes and expressing the specific enzymes required for their different roles within the tissue. Teleosts, including zebrafish, are among the very few vertebrate organisms that possess the ability to regenerate many organs and tissues following amputation or injury during adulthood (Akimenko *et al*., 2003; Poss *et al*., 2003). The tail fin of teleost is composed of structural units named rays (fin rays) or lepidotrichia, the skeletal elements supporting the fin structure. It is acellular and each lepidotrichium has series of bony segments comprised of a pair of concave hemirays joined end to end by ligaments and surrounded by a monolayer of scleroblasts (bone-secreting cells). The space between two hemirays is filled with connective tissue and contains nerves, blood vessels, pigment cells, and fibroblasts. Finally, the lepidotrichia are surrounded by a multi-layered epidermis (Poss *et al*., 2003). The lepidotrichium is filled with an extracellular matrix containing type II collagen fibrils of different orientations surrounded by mineralized ground substance rich in chondroitin sulfate (Montes *et al*., 1982). Rays are connected to each other through a soft inter-ray region, which is composed of connective tissue and epidermis. Each ray ends distally with two palisades of rigid but non-mineralized fibrils named actinotrichia. Actinotrichia are small, rigid, fusiform spicules found in brush-fashioned groups present distally in rows (Ryder, 1885; Mari Beffa *et al*., 1989) and parallel to each hemi-ray inner face. The actinotrichia are formed from hyperpolymerised macrofibrils of elastoidin, a protein with similar characteristics to those of collagen (Gross and Dumsha, 1958; Mari-Beffa *et al*., 1989) and collagens are associated with non-collagen components. The non-collagen components of actinotrichia (actinodins) have been shown to play a critical role in the fin to limb transition. Recent studies have reported the

morphogenetic role of actinotrichia in fin formation and the same report also suggested that bone morphogenetic protein (BMP) represents the first known signaling that induces actinotrichia formation in the regenerate (Thorimbert *et al*., 2015).

When partially amputated or severely injured, fins are able to complete self-restoration through the process of epimorphic regeneration (Géraudie and Singer, 1992). A series of regenerative stages are initiated following amputation of caudal fin and complete restoration of all lost tissues occurs *via* epimorphic regeneration in approximately 2 weeks in adult fish (Tal *et al*., 2010). Epimorphic regeneration is achieved by following stages: wound healing (12-24hpa), blastema formation (24-48hpa) and regenerative outgrowth (48hpa-2 weeks) (Yoshinari et *al*., 2009). Upon removal of the caudal fin, an epithelial layer covers wound by 24hpa. By 48hpa, blastemal cell population undergoes intense proliferation and then dedifferentiates into a variety of cells which ultimately culminate into the regenerated fin. Dedifferentiation program involves the formation of connective and skeletal tissues which begins with scleroblast formation from blastemal cells in contact with epidermal layer (Smith *et al*., 2006). These scleroblasts synthesize and secret dermal bone matrix and accumulate in sub-epidermal space. Finally, it mineralizes and bone tissue is reformed in amputated fin. BMP signaling can be one of the signaling pathways directing bone regeneration in caudal fin. Several other signaling pathways have also been found to be required for fin regeneration, including Wnt, FGF, Activin, IGF, Retinoic acid and Sonic hedgehog (Stoick-Cooper *et al*., 2007a; Poss, 2010; Tal *et al*., 2010). Although we are far from achieving a thorough understanding of the precise roles these pathways play in fin regeneration. All of these signals have been shown to be required for proliferation of the progenitors of the blastema. However, little is known about the signals that regulate differentiation during epimorphic regeneration.

Bone morphogenetic protein, a family of 20 members participates in a variety of processes during embryogenesis, tissue development and tissue repair (Boergermann *et al*., 2010). BMPs also play a major role in skeletal development, bone formation and mesenchymal stem cell (MSC) differentiation (Kang *et al*., 2004). MSCs are adult stem cells having the unique ability to self-renew and to differentiate into various mesodermal cell lineages, osteoblastic, chondrocytic, myocytic and adipocytic, as well as non-mesodermal tissues, such as cardiac muscle and skin (Aubin, 2001; Kang *et al*., 2004; Luu *et al*., 2007; Deng *et al*., 2008; Kang *et al*., 2009; He *et al*., 2010). The differentiation of these multipotent stem

cells towards an osteoblastic fate depends on numerous signaling pathways, including BMP transduction (Figure 3.1).

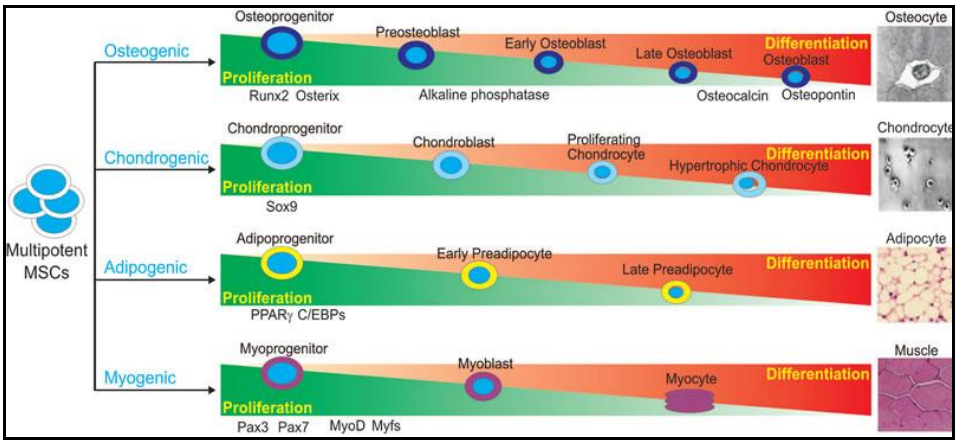

Figure 3.1: Schematic representation of lineage-specific differentiation of mesenchymal stem cells. Regulators and indicators of lineage-specific differentiation are depicted here (from Beederman *et al*., 2013).

The osteoinductive ability of BMPs was discovered when it was found that demineralized bone could induce *de novo* bone formation by BMP-induced osteogenesis (Urist, 1965; Wozney *et al*., 1988). Disruptions in BMP signaling have subsequently been shown to result in a variety of skeletal and extra-skeletal anomalies (Hogan, 1996c; Zhao, 2003). At least 15 different BMPs have been identified in humans to date. BMP-9 is among the most osteogenic BMP and promotes the osteoblastic differentiation of mesenchymal stem cells both *in vitro* and *in vivo* (Cheng *et al*., 2003; Peng *et al*., 2003; Kang *et al* 2004; Luo *et al*., 2004; Peng *et al*., 2004; Luu *et al* 2007).

Mesenchymal stem cells undergo several stages of maturation during their proliferation and differentiation along the osteoblastic lineage. MSCs initially form preosteoblasts, which proliferate near the surface of the bone and secrete alkaline phosphatase, an early marker of osteogenesis (Reddi, 1997; Lian *et al*., 1998; Karsenty, 1999; Luu *et al*., 2007). Preosteoblasts further mature into osteoblasts, which are involved in initial extracellular matrix maturation and mineralization. Osteoblasts ultimately form mature osteocytes which are terminally differentiated cells embedded in an extracellular matrix responsible for mechanical support and regulating the mineralization of bone (Lin and Hankenson, 2011; Marcellini *et al*., 2012). These stages of osteogenic proliferation and differentiation are characterized by expression of various markers, including cell-cycle associated genes during

the proliferative phase, the early osteoblastic marker- alkaline phosphatase and late markers- Osteocalcin and Osteonectin, connective tissue growth factor (CTGF), inhibitor of DNA binding (Id) and cbfa1/runx2 (Yamaguchi *et al*., 2000; Peng *et al*., 2003; Luo *et al*., 2004; Peng *et al* 2004; He, 2005; Lian *et al*., 2006; Luu *et al*., 2007; Kang *et al*., 2009). From MSCs, bone can form in one of two ways, either by endochondral or by intra-membranous ossification (Olsen *et al*., 2000). In endochondral ossification, cartilage is formed as a precursor on which new bone is laid down whereas, during intra-membranous ossification, direct laying down of bone into the primitive connective tissue (mesenchyme) occurs. There is no intermediate cartilage involved during intra-membranous ossification. Thus, bone maintenance and remodeling depends, in part, on the proper formation of osteoblasts from MSCs, a highly regulated and complex process in which the BMP signaling pathway plays a critical role.

Further, knockout of specific BMPs or mediators of BMP signal transduction often leads to phenotypes which demonstrate the critical importance of BMP signaling in skeletal development. BMP signaling is required for differentiation of multi-potent mesenchymal cells into osteochondroprogenitor cells, which are capable of forming both chondrocytes and osteoblasts. BMP signal transduction is also necessary for the proper functioning of differentiated osteoblasts, enabling them to appropriately secrete the matrix upon which bone formation occurs (Karsenty, 1999).

The first genetic evidence that BMPs have a role in bone morphogenesis was through natural loss-of-function mutations of BMP-5, resulting in short ear phenotype and brachypodism (King *et al*., 1994; Zhao, 2003). Several studies have also investigated mediators and inhibitors of BMP signal transduction. Noggin is a well-established antagonist of BMPs, and overexpression of noggin results in osteopenia (Smith and Harland, 1992; Valenzuela *et al*., 1995; Devlin *et al*., 2003; Canalis *et al*., 2003). Other studies have also shown that bone and skeletal development are decreased when BMP antagonists are overexpressed in osteoblasts (Devlin *et al*., 2003; Wu *et al*., 2010).

BMP signaling plays a role in fin growth and bone formation during zebra fish fin regeneration. BMP-2, 4 and 6 are involved in differentiation of chondrocytes to form cartilage (Minina *et al*., 2001; Yoon and Lyons, 2004) and both differentiation and maturation of cells of osteoblastic lineages which forms bone (Yamaguchi *et al*., 1991;

Thies *et al*., 1992; Rickard *et al*., 1994; Canalis *et al*., 2003; Wan and Cao, 2005). BMP-2 is considered one of the most potent extracellular signaling molecules that stimulate bone formation and osteoblast differentiation (Choi *et al*., 2005). BMP-2b and probably BMP-6 participate in zebrafish caudal fin regeneration by regulating the expression of sox9a, runx2a, runx2b and col10a1 (Smith *et al*., 2006).

Cell death plays a role in sculpting the limb. Indeed, it is essential if our joints are to form and if our fingers are to become separate (Zaleske, 1985). The death (or lack of death) of specific cells in the vertebrate limb is genetically programmed and has been selected for during evolution. During the differentiation or growth stage programmed cell death is a vital mechanism which regulates the cell survival and homeostasis. Roles of cell death in sculpting of growing tissue has been reported in a number of developmental systems including, heart, limb and craniofacial patterning (James, 1994; Guha *et al*., 2002; Bastida *et al*., 2004), as well as nervous system pruning (Erdem *et al*., 1998; Honig and Rosenberg, 2000; Bagri *et al*., 2003; Johnston, 2004). Recent reports have also indicated that suppression of apoptosis plays an essential role in stem cell self-renewal (Domen and Weissman, 2000; Domen *et al*., 2000; Opferman *et al*., 2005; Yamane *et al*., 2005). BMPs (along with FGF) are known to be involved in apoptosis as well as proliferative activities and a controlled regulation of these factors and the associated processes are required for successful regeneration. Recent findings show that Drosophila legs are also sculpted by programmed cell death. In this case, rather than the absolute activity of BMP, it is the sharp discontinuity of BMP signaling that is required for forming the leg joint. BMPs are known to be expressed in the inter-digital zones of the developing limbs in vertebrates, where they trigger apoptotic signals (Milán, 2007).

As demonstrated previously (chapter 1 and chapter 2) the administration of LDN193189 results in down-regulation of dlx5 and runx2 genes in 24hpa and 60hpa tissues of treated fishes. These two genes are direct downstream targets of BMP signaling. This way we validated BMP signaling inhibition by LDN193189. Ablating BMP signaling may outline the mechanism behind orchestrating bone and cartilage regeneration in teleost fish. LDN193189, a derivative of Dorsomorphin is able to block the activation of Smad1/5/8 as well as the non-Smad pathways through MAPKs p38, ERK1/2, and the Akt pathway by targeting BMP receptors (Boergermann *et al*., 2010).

Therefore, the objective of the current study was to observe the possible effects of BMP signaling inhibition on early differentiation stage (5dpa) of regeneration in *Poecilia latipinna*. Herein, the effort was made to study various cellular activities and patterning of actinotrichia in the tail fin after hampering the BMP signaling by administering LDN193189.

MATERIAL AND METHODS

Animal maintenance and tissue collection

As detailed earlier in the general material and methods section, all the animals were maintained in suitable conditions and collections were done on the 5^{th} day of amputation. Collected tissues were used for the experiments listed below.

Morphometric analysis

The length of the regenerating fin was measured on 5^{th} day post-amputation using digital vernier caliper. Fin length was measured from the level of amputation plane to the distal end of the regenerate and images were also captured through a microscope.

Histology

The fins were dehydrated through an ascending ethanol series, and embedded in paraffin. Longitudinal sections (8-10µm) were cut and stained with Hematoxylin and Eosin (H&E), to observe the general structural changes if any in regenerated fin .The sections were mounted in DPX mountant.

SDS-PAGE

Regenerating tail fin tissue from each experimental group was amputated 5dpa, 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.

Skeletal preparation

Whole mount staining of fins was performed by Alcian blue and Alizarin red staining method as described by Ferretti and Géraudie, (1995) and Laforest *et al*. (1998).

Hydroxyproline assay

As a total measure of collagen in the regenerated tissue 5dpa, hydroxyproline content was estimated by a method proposed by Edwards and O'Brien (1980).

Gene expression analysis by qRT-PCR

Expression of dlx5, runx2, sox9, Osteocalcin (Osc), Osteonectin (Osn) and col10a1 was analyzed by quantitative RT-PCR. The technical details can be found in materials and methods. Primer sequences are listed in Table 3.1.

Table 3.1. Primer sequences for Osteocalcin, Osteonectin, col10a1 and sox9

Gene	Forward primer	Reverse primer	T_a (°C)	Product size (bp)
Osc	CCCAACTGTTGAGGAACCAGA	TGTCATCGACCTCACACACC	59	152
Osn	GGGGAAGGGAGCTGACTTAG	GCAGCATCTGAAGTCACGTAG	60	170
col10a1	CTTCACGGTTTAGCTTCACAAG	TTGTGGTCCTGCAACAGAGT	59	154
sox9	CAGACGCACATCTCTCCGAA	TCCCTGTGACTGACCCGAAT	60	114

Immunohistochemistry of Caspase-3

Whole-mount immunohistochemistry was performed to detect activated Caspase-3 activity. Briefly, Zone of interest was collected 5dpa for IHC and was fixed in 4% paraformaldehyde prepared in PBS for 4hr. After fixing the tissue was washed in PBS for 5minutes and then processed as per protocol described earlier in materials and methods.

RESULTS

BMP signaling inhibition impedes outgrowth of the regenerating fins

To investigate the effects of BMP signaling inhibition on fin regeneration, regenerated fin length and ray length was measured at 5dpa. A noticeable length difference ($p \leq 0.001$, n=16) in LDN193189 treated and control fins was recorded (Figure 3.1). The histological analysis of H&E stained tissues revealed that the fins treated with LDN193189 began to exhibit significantly delayed regeneration in comparison to those in the control group (Figure 3.2). LDN193189 treated fins showed non-continuous epithelial layer compared to control. Additionally, bending of actinotrichia was also observed in fins of the animals treated with LDN193189 (Figure 3.3).

Protein profile of early differentiation stage is altered due to LDN193189 treatment

The cell differentiation process leading to regeneration involves a major change in the expression pattern of peptides from the resting stage. Whether inhibition of BMP signaling affects the expression of peptides in any way during the early differentiation stage of regeneration in *Poecilia latipinna*, was studied using SDS-PAGE. The intensity of almost all polypeptide bands was augmented after LDN193189 treatment. The bands with the most significant increase in intensities were in the molecular weight range approximately 80 and 29kDa (Figure 3.4; Table 3.2).

Inhibition of BMP signaling delays bone formation

In higher vertebrates, BMP signaling is shown to regulate bone and cartilage formation. To get insight into the extent of bone and cartilage of whole fin tissues of control and LDN193189 treated were stained with Alcian blue and Alizarin red. Alizarin red positive regions did not appear in the regenerating part of the fin. Alcian blue-positive chondrogenic regions appeared in the distal fin region of both group animals which suggested the presence of cartilaginous precursor cells in the regenerating ray (Figure 3.5), hence we targeted sox9 expression which is chondrocyte marker (Figure 3.6). The results revealed the down-regulation of sox9 expression in LDN193189 treated fins during early differentiation stage. To refine the histological studies, a molecular aspect of BMP signaling in bone and cartilage formation was also examined. Smad-dependent BMP signaling triggers, activate and regulate expression of transcriptional regulators (dlx5 and runx2) as well as skeletogenesis markers (Osn and Osc) important in skeletal cells. Quantitative Real-time PCR confirmed momentous down-regulation of dlx5, runx2, Osn and Osc (Figure 3.6).

Ablation of BMP signaling results in deformed connective tissue

The H&E staining results indicated noteworthy changes in the connective tissue of regenerating fin for LDN193189 treated fins compared to control fins. To shed more light on the obtained results, collagen-X was targeted as representative of ECM component. Collagen contains hydroxyproline amino acid in its structure and hence we estimated hydroxyproline content in LDN193189 treated and control fins. Hydroxyproline content showed a considerable reduction in treated fins (Figure 3.7). Further to validate the current results we examined col10a1 transcript levels in both control and LDN193189 treated fin tissues (Figure 3.6). The results did match with hydroxyproline content and histology

outcomes indicating that LDN193189 impairs not only skeletogenesis but also connective tissue formation during lepidotrichial regeneration.

Programmed cell death is augmented by BMP signaling inhibition

To check the apoptotic activity during early differentiation stage (5dpa) in *Poecilia latipinna*, Caspase-3 enzyme was localized by immunohistochemistry. As observed in Figure 3.8, Caspase-3 activity started appearing near the amputation plane and was increased progressively by several times towards the distal fin edge in the fins of LDN193189 treated animals compared to the fins of the control group animals where apoptotic activity was limited to the fin edge only. Additionally, Caspase-3 activity was also observed in the inter-ray tissue of treated fins. Overall, it showed an unusual pattern of apoptosis due to diminished BMP signaling in treated fins.

DISCUSSION

Out of the variety of functions performed by BMPs, cell proliferation, and connective tissue formation was brought into focus in this study. We inhibited BMP signaling by LDN193189 in the regenerating fin of *Poecilia latipinna*. Our aim was to observe the effect of inhibition of BMP signaling during early differentiation stage (5dpa). We began our studies with morphometric analysis of regenerating fin. Due to LDN193189, fin length and ray length was significantly decreased. Earlier studies suggested that misexpression of chordin leads to a drastic reduction in ray length of regenerating fish fin in zebrafish (Smith *et al*., 2006). Moreover, the histological profile of treated fins showed deformed connective tissue and epithelial layer compared to control fins. To find out reasons behind this, we looked into molecular as well as structural details of regenerating fins. To begin with a molecular aspect, we studied the change in protein profile at this stage. A number of proteins synthesized during differentiation stage expressed were common in both the groups. A spot densitometric scan of the PAGE gels revealed up-regulation of almost all the peptides. The bands with the most significant increase in intensities were in the molecular weight range approximately 80 and 29kDa.

Further, to elucidate the role of BMP signaling in skeletal development, bone and cartilage tissue formation was undertaken in the current study. BMP signaling is known to regulate bone and cartilage formation through discrete cascades. BMP-2 and BMP-7 are reported to show osteogenic effects (Wozney, 1992). We performed Alcian blue and Alizarin red

staining in treated and control fins and observed only cartilaginous tissue in the distal end of treated fins and no bone cells. To get molecular minutiae behind this, we embattled downstream targets of BMP signaling and found both dlx5 and runx2 gene were down-regulated in treated animals compared to control. Gersbach and coworkers (2004) have reported that dlx5 and runx2 are induced by BMP signaling in chick primary calvaria cells. Further, it has been documented that dlx5 drives runx2 expression and osteogenic differentiation in osteogenic mesenchymal cells (Holleville *et al*., 2007). Besides, Osteonectin and Osteocalcin gene expression was also declined notably. Osteonectin and Osteocalcin are late differentiation markers involved in bone mineralization (Sousa et al., 2011). Altogether our results suggested a delay in bone differentiation in treated fins due to ablated BMP signaling.

Early differentiation also includes connective tissue formation. 5dpa sections of control and treated fins were stained with H&E stain to visualize the structural integrity of regenerate. Results showed discontinuous epithelium in LDN193189 treated tissue which indicates defective ECM formation. Collagen is one such component of ECM which serves morphogenetic role during teleost fin regeneration (Marí-Beffa *et al*., 2003). The col10a1 expression was detected in newly differentiating scleroblasts and basal layer of epidermis during zebrafish fin regeneration (Smith *et al*., 2006). In our studies, gene expression analysis suggested a drop off by 0.013 fold change for col10a1 in treated fins. Reduction in collagen content in treated fins was also recorded in the supplementary biochemically analysis. Moreover, morphological observation revealed disorganized and condensed collagen fibrils in newly formed actinotrichia of LDN193189 treated animals. BMP represents the first known signaling that induces actinotrichia formation in the regenerate (Thorimbert *et al*., 2015). The present results, therefore, clearly establish that BMP signaling inhibition could be the reason behind observed structural deformities in tail tissues of fishes that received LDN193189.

There are two major consequences following BMP signaling inhibition, drastic delay in growth of regenerate and the malfunctioning of the bone matrix-secreting scleroblasts (Smith *et al*., 2006). Both of the phenomena were explored in the study in early differentiation stage. LDN193189 could inhibit regenerative outgrowth of the caudal fin at 5dpa. As regeneration was at a halt, none of the subsequent processes took place. The Smad-dependent BMP signaling regulates bone and cartilage formation. One such

prerequisite transcription factor involved in bone formation is runx2. The runx2 is a known transcriptional regulator of chondrocyte maturation and osteoblast differentiation (Harada and Rodan, 2003). In the current study, a significant reduction in the expression of runx2 was recorded in the LDN193189 treated group. This decrease in runx2 expression might have inactivated scleroblast differentiation and function in the regenerating caudal fin of *P. latipinna.* It has been reported that type X collagen gene (col10a1) is a direct downstream target of runx2 in mouse, chick and human (Smith *et al*., 2006). Hence, the col10a1 expression was studied and a significant reduction was noted in the samples collected form treated fishes compared to that of controls. In addition, Osteocalcin and Osteonectin two bone-specific extracellular matrix proteins and markers of mature osteoblasts were also quantified at the transcript level. LDN193189 treated animals displayed a significant decrease in Osn and Osc gene expression during early differentiation. Altogether, the above results suggest that during early differentiation stage there is a delay in bone differentiation in treated fins due severe down regulation of molecular cues for osteogenic differentiation due to ablated BMP signaling.

During the growth of any tissue, a finely balanced mechanism between cell proliferation and cell death acts to ensure the accurate development of that particular tissue. Programmed cell death is known to be involved in the sculpting of growing tissues in different development systems. Early reports have suggested the signal for apoptosis in the autopod is probably provided by the BMP proteins. BMP-2, BMP-4, and BMP-7 are reported to express in the interdigital mesenchyme and blocking BMP signaling prevents inter-digital apoptosis (Yokouchi *et al*., 1996; Zou and Niswander, 1996). Hence, we suspected a role of BMP in fin patterning during regeneration and to establish a relationship between BMP signaling and apoptosis, if any, Caspase-3 was localized in the fin sections. Data revealed significantly increased the activity of Caspase-3 enzyme in LDN193189 treated group. The increased activity of Caspase-3 near the distal part of the fin can be correlated to the discontinuous epithelialization of the fin regenerates of LDN193189 treated animals. These results suggest that blocking BMP signaling during early differentiation stage promotes apoptosis in tail fin of *Poecilia latipinna*. To comprehend the current findings, BMP signaling retardation during early differentiation of regeneration in *P. latipinna* delays bone formation and causes deformities in connective tissue. Moreover, inhibition of BMP signaling breaks the equilibrium between cell proliferation and apoptosis and promotes more cell death in LDN193189 treated animals which culminates in stalled regeneration.

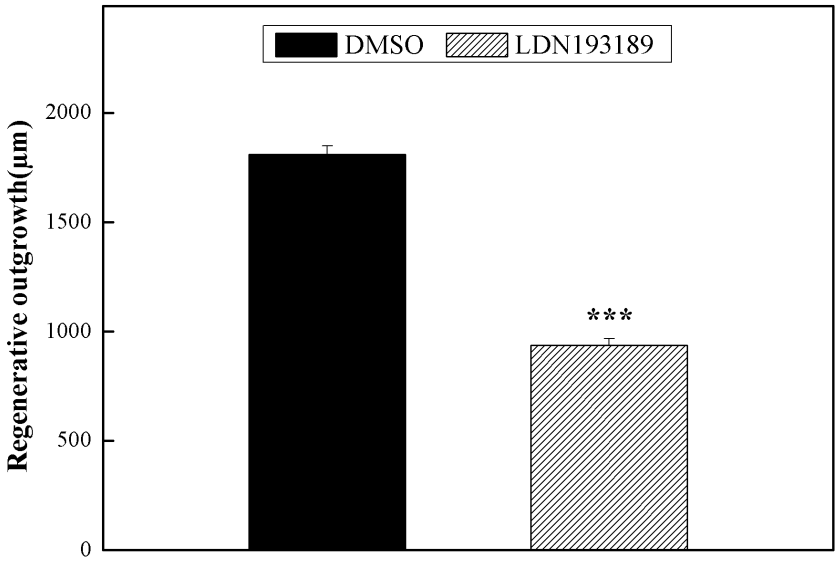

Figure 3.1: Morphometric analysis of caudal fin regenerate at 5dpa. Fish were treated with DMSO or 2.5mg/kg body weight LDN193189.The treated group demonstrated significant reduction in regenerating tissue ($P \leq 0.05$, Student's t-test) compared to control group. Mean length of fin regenerates (n=8); ***P<0.001

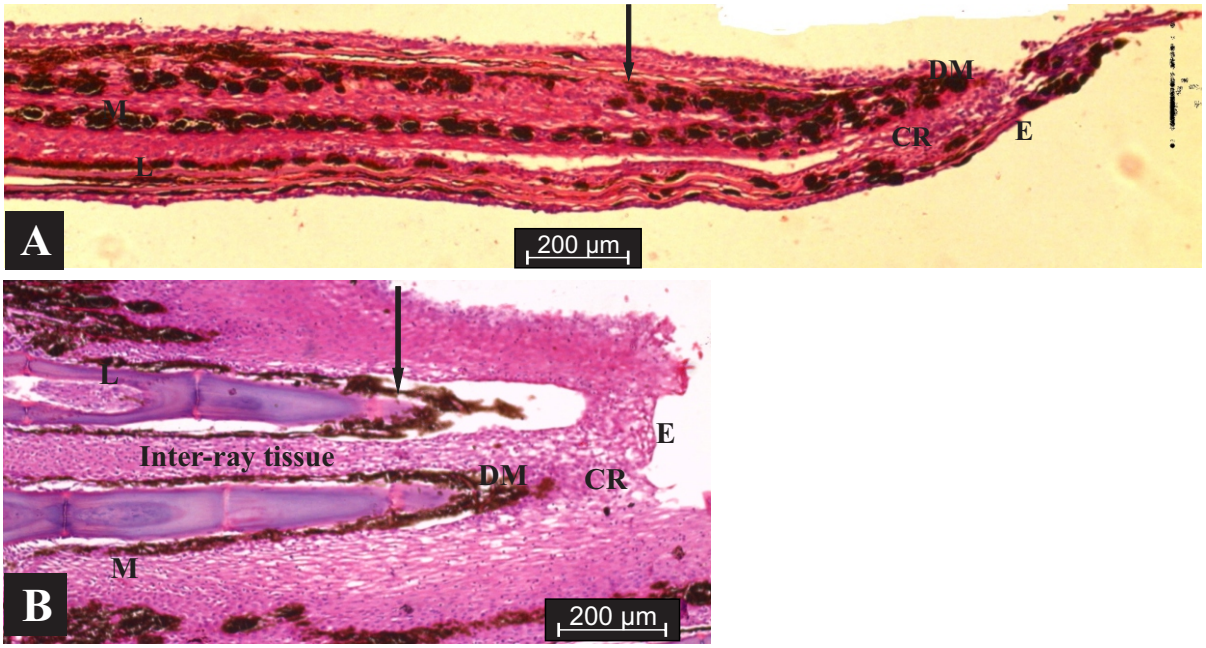

Figure 3.2: Histology profiles: Longitudinal Section of the distal region of the caudal fin of fish at 5dpa of regeneration from control (A) and LDN193189 (B) treated group. Black arrows indicate approximate level of amputation.

E: Epidermis, L: Lepidotrichia, CR: Connective tissue in regeneration, M: Melanocyte, DM: Differentiated Melanocyte.

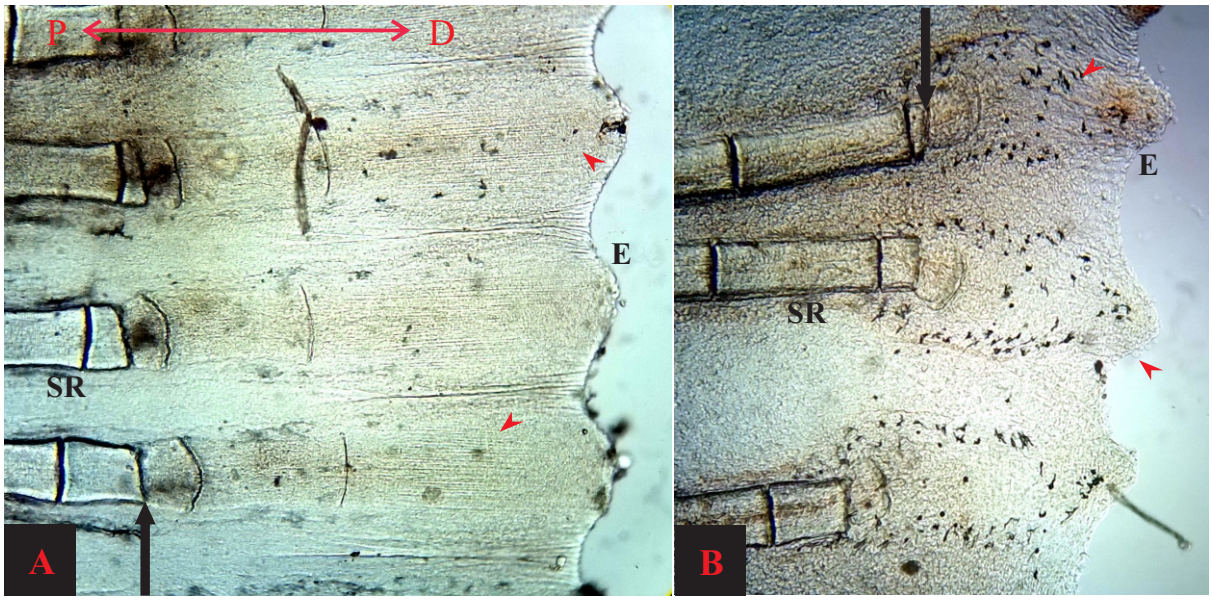

Figure 3.3: Morphological structure of caudal fin at 5dpa. Fin regeneration is subdued by LDN193189 treatment (B) when compared to DMSO control (A). Black arrows indicate amputation plane. Usual array of actinotrichia (A) and unusually reduced tissue growth and array of actinotrichia after LDN93189 treatment(B)shown by red arrowheads.

E: Epidermis, SR: Segmented ray, P: Proximal , D: Distal direction; Magnification 10X

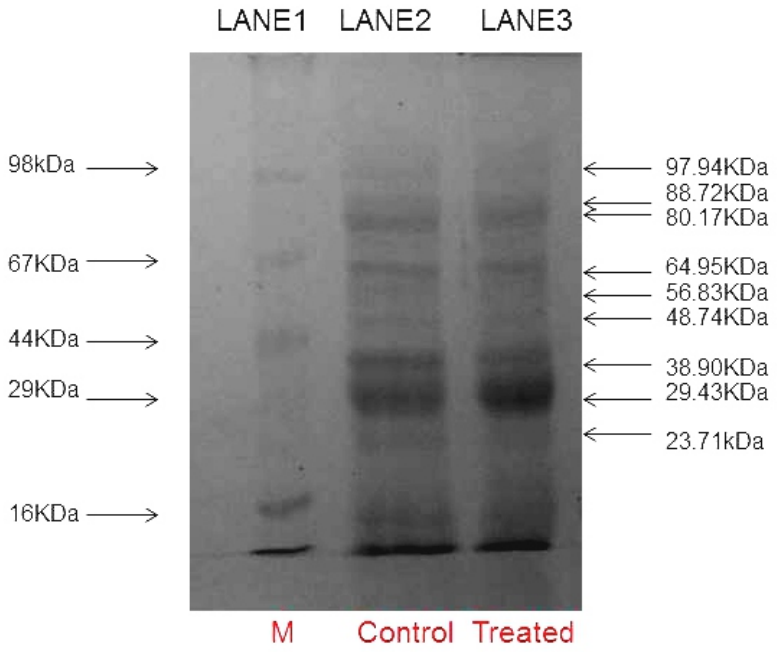

Figure 3.4: PAGE picture of protein extracts from caudal fins of control and LDN-treated fish at 5dpa. Peptides showing significant difference in expression between control and treated samples have been labeled with their molecular weights

Molecular weight (approx in kDa)	Relative band intensity (in arbitrary units)	
	Control	Treated
97.94	16.65	26.39
88.72	21.99	30.08
80.17	2.28	43.26
64.95	31.63	49.27
56.83	36.42	44.21
48.74	35.42	45.45
38.90	47.43	56.58
29.43	66.16	90.26
23.71	49.59	56.79

Table 3.2: Relative band intensities of peptides in control and LDN193189 treated group fins at 5dpa.

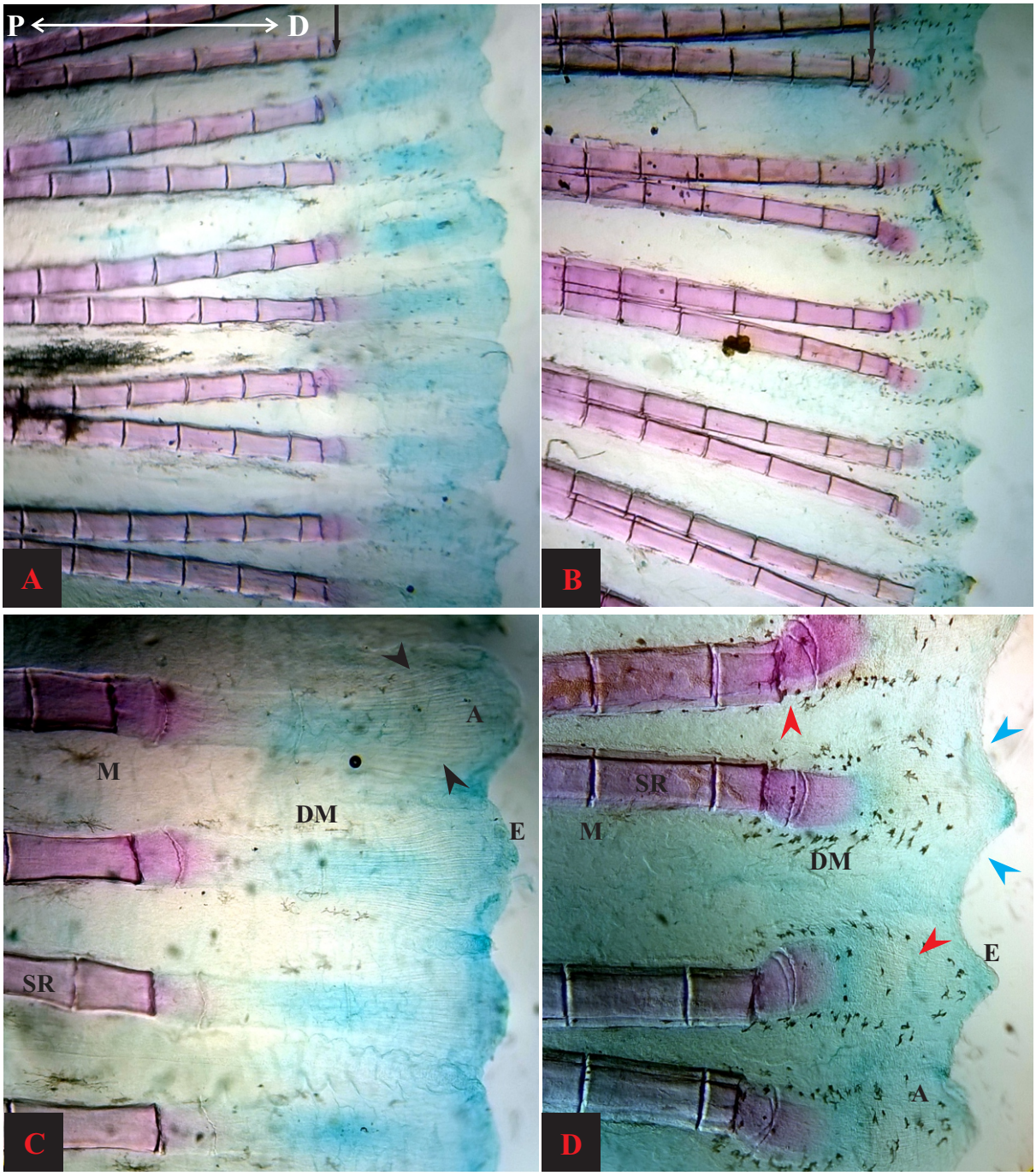

Figure 3.5: Fin elements stained for cartilage and bone at 5dpa using Alcian Blue and Alizarin Red respectively,

Control (A and C) and LDN193189 treated (B and D). Black arrowhead indicates normal actinotrichia.LDN193189 induced abnormalities in regenerating fin is indicated by red arrowheads. Blue arrowheads indicate unusual pattern formation at distal regenerating fin end. Black arrows indicate amputation plane. Black arrows indicate plane of amputation.

E: Epidermis, A: Actinotrichia, SR: Segmented ray, M: melanocyte, DM: Differentiated Melanocyte, P: Proximal , D: Distal direction. Magnification: 4x (upper panel) and 10x (lower panel).

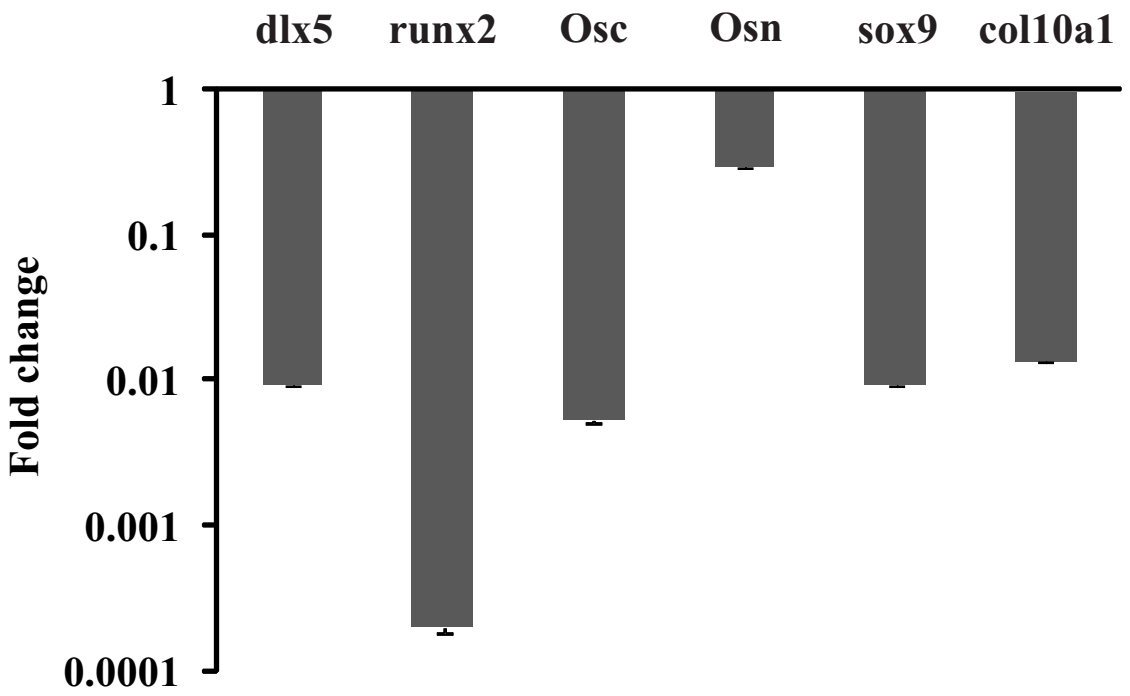

Figure 3.6: Mean fold change ($2^{-\Delta\Delta Cq}$) in expression of genes involved in connective tissue formation in LDN193189 treated and control animals. Error bars represent standard error mean

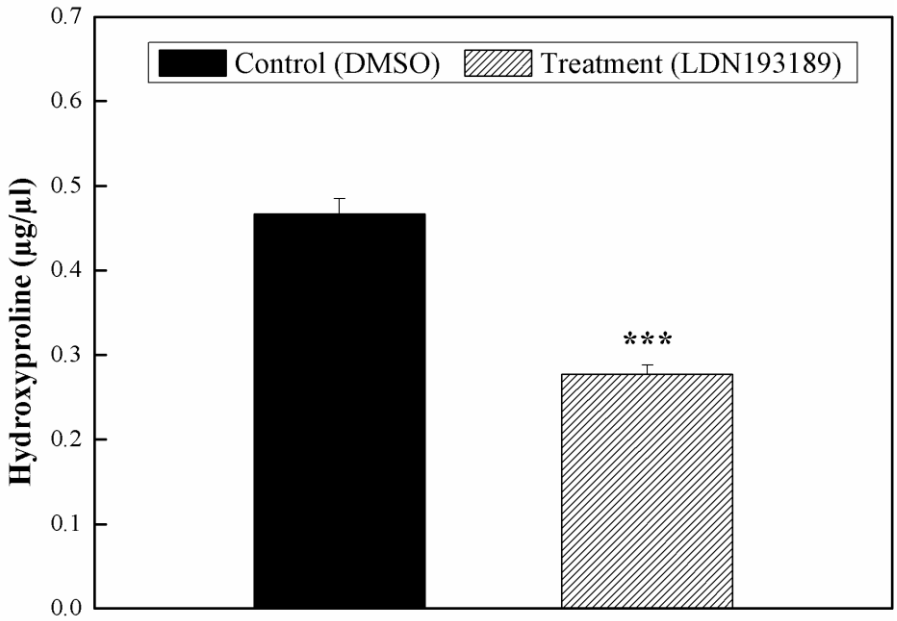

Figure 3.7: Hydroxyproline estimation as a measure of collagen. The collagen production of fin tissues was significantly decreased (***P < 0.001, Student's t-test) in the fins of LDN193189 treated group animals in relation to fins of the control group animals.

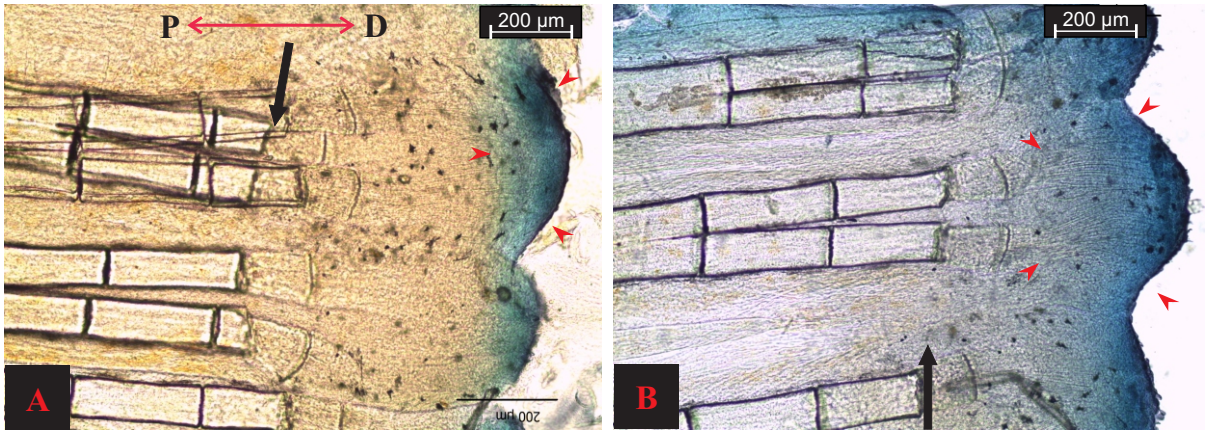

Figure 3.8: Immunohistochemical detection of activated Caspase-3 at 5dpa during fin regeneration.Control fin (A) LDN193189 treated fin (B). Black arrows indicate amputation plane. Red arrow-heads indicate areas of apoptosis.