GENERAL CONSIDERATIONS

Many lowly evolved animals can re-grow or restore their lost appendages however, with the exception of terminal phalanges of neonates, mammalian appendages do not regenerate. Is there something the mammals (including human) can acquire from these animals? The connection among regeneration, life and death has been curtly summed up by a pioneer in the field of regenerative biology, an outstanding educator and a consummate naturalist, Professor Richard J. Goss (1969) in the following words "If there were no regeneration there could be no life. If everything regenerated there would be no death. All organisms exist between these two extremes. Other things being equal, they tend towards the latter end of the spectrum, never quite achieving immortality because this would be incompatible with reproduction".

The restoration of lost body parts and injured organs always attracted the human imagination ever since the time of ancient Greeks. The desire to induce human tissue regeneration has been revived by a number of recent developments. Regenerative medicine "rebuilding organs and tissues" could conceivably be the 21st century equivalent of antibiotics of the 20th century. The important thing here is to understand the signals that control regeneration. Researchers have been puzzled for centuries over how body parts replace themselves. In the mid 1700s, for instance, Swiss researcher Abraham Trembley noted that when chopped into pieces, hydra could grow back into complete, new organisms. Other scientists of the era examined the salamander's ability to replace a severed tail along with lizard's, as a reptilian model and a century later, Thomas Hunt Morgan scrutinized planaria, flatworms that can regenerate even when whittled into 279 bits.

Animals exploit three prime approaches to regenerate organs. First, cells that normally do not divide can multiply and grow to replenish the lost tissue, as occurs in injured salamander heart. A second approach, whereby specialized cells regress to a simpler state, reminiscent of stem cells (a process known as dedifferentiation), that can replicate and later re-specialize when supplied with the appropriate factors to reconstruct a missing part. Some of the amphibian members such as Urodeles take this approach to heal and rebuild a severed limb, as do zebrafish to repair clipped fins and lizard to restore its lost tail. Finally, pools of stem cells step in to perform required renovations when required. Planarians tap into this resource when reconstructing themselves (Gilbert, 2000).

Nonetheless, the process of regeneration is generally divided into two types, *viz.*, morphallactic and epimorphic regeneration. In morphallaxis, majority of regenerated tissue comes from already present tissue in the organism through reorganization. Morphallaxis is often contrasted with epimorphosis, which is characterized by a much greater relative degree of cellular proliferation although cellular differentiation is active in both processes. The epimorphic regeneration involves the generation of new stem cells, either by proliferation of the existing stem cells or by reprogramming (dedifferentiation) of the adult cells, which redifferentiate to different cell types to form the lost appendage which is of a size similar to that of the original. However, only few classes of lower vertebrates show true epimorphic regeneration like Urodeles and Anurans. More precisely, Urodele amphibians are able to regenerate an impressive repertoire of complex structures including parts of their hearts, eyes, spinal cord, tail, and they are the only adult vertebrates able to regenerate full limbs (Gardiner *et al*., 2002).

Generally, the regeneration process starts with wound healing, followed by the formation of a blastema of proliferating cells that go on to form a complete array of tissue types. Unwinding the mysteries of regeneration will depend on understanding what separates the mammalian wound healing process from that of other animals that are able to regenerate. The difference might be subtle. Researchers have identified one strain of mice that seals up ear holes in weeks, whereas typical strains never do. A relatively modest number of genetic differences seem to underlie the effect. Perhaps altering a handful of genes would be enough to turn us into super-healers, too. But if scientists succeed in initiating the process in humans, new questions will emerge. In recent years the emphasis in studying vertebrate limb/tail regeneration has shifted from the identification of requirements for regeneration to the characterization of the mechanisms that control the formation of the lost tissue. The requirements of regeneration have been characterized on the basis of inhibition studies. This concept has revealed some of the necessities for proper regenerative response, *viz.*, innervations, cytokines, growth factors, neural and hormonal factors, etc (Han *et al*., 2013).

The process of regeneration is a complex one, possessing a number of distinct phases (Abdel-Karim *et al*., 1990; Cadinouche *et al*., 1999; Gardiner *et al*., 2002). During epimorphosis, wound healing is a critical process where multiple cells types, soluble mediators and extracellular matrix (ECM) components are involved. Instructions governing all these processes in a coordinated manner result in appropriate regeneration of the tissues. Inflammatory cells (macrophages, neutrophils, lymphocytes) play a significant role not only in the coordination of the overall inflammatory response to injury/trauma encountered after amputation, but also in directing the successive activity of mesenchymal and epithelial cells (Balemans and Van Hul, 2002). All these processes involve a precise and controlled regulation of signaling molecules. Signaling molecules are essential proteins that play a critical role during development as well as regeneration like Wnt, Notch, Shh, FGFs, BMPs, etc. BMP is one such signaling molecule that plays an imperative role during epimorphic regeneration. Bone morphogenetic proteins (BMPs) are a group of growth factors also known as cytokines and as metabologens, belonging to the transforming growth factor β (TGF-β) super-family, whose signaling regulates many aspects of skeletal development and which play important roles in embryonic growth and patterning in both vertebrates and invertebrates (Graff, 1997; Hogan, 1996a; Balemans and Van Hul, 2002; Wan and Cao, 2005). More than 40 types of BMPs have been described (Matthews, 2005). 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; Massagué, 2000; Wozney, 2002; Yamamoto and Oelgeschläger, 2004). Although the morphogenetic and cellular aspects of appendage regeneration have already been widely studied in several parts of the world, the molecular basis underlying the process still remains only partially understood. For these reasons, it seems appropriate to address the role of BMP signaling in teleost caudal fin regeneration.

BMPs signal through a coordinated assembly of two types of trans-membrane serine/threonine kinase receptors, heteromeric complex composed of type I and II subtypes. After ligand binding, the type II receptor phosphorylates and activates the type I receptor. Three type I receptors have been shown to bind BMP ligands, type IA and IB BMP receptors (BMPR-IA or ALK-3 and BMPR-IB or ALK-6) and type IA activin receptor (ActR-IA or ALK-2) (Wan and Cao, 2005). Signal transduction studies have revealed that Smad 1, 5 and 8 are the immediate downstream molecules of BMP receptors, they then form a complex with co-Smad, Smad4. Later Smad1/5/8 complex translocates to the cell nucleus and functions as transcription factor (Zwijsen *et al.*, 2003), playing a central role in BMP signal transduction. Phosphorylation of these specific Smads results in various cellular effects, including growth regulation and differentiation. Signaling via BMP receptors may also activate other pathways, including mitogen activated protein kinase (MAPK).

Considering the diverse roles, as discussed earlier, played by the BMP signaling during embryonic development, it was presumed that BMP signaling could be a major modulator of epimorphic regeneration in vertebrates. In order to test this hypothesis the current study was designed wherein the role of BMP signaling in achieving various stages of appendage regeneration was attempted in an accomplished vertebrate model for regeneration - the tail fin of teleost fish *Poecilia latipinna*. For the preliminary study the BMP receptors were targeted to inhibit the BMP signaling in the selected animal model. The simple assumption behind it was if a chemical inhibits or modulates an essential molecular target, then regeneration will be impacted. The identification of the chemical target will thus help to disclose the underlying molecular pathways that permit tissue regeneration. Though there are many methods to block the BMP signaling, inhibition of pathway via synthetic inhibitor was preferred. One of the novel indolinone derivatives named LDN193189 was selected for the study since it is a more potent and selective inhibitor of BMP signaling than other available inhibitors like dorsomorphin (Kamiya *et al.*, 2010). And also, LDN193189 is reported to be more selective in targeting BMP signaling without interfering with VEGF or TGF-β signaling (Kamiya *et al.*, 2010).

Studies have shown that the amputation of the caudal fin leads to a succession of steps, namely wound healing, blastema formation and regenerative outgrowth, that restore the various tissues of the fin, including blood vessels, nerves, connective tissue, epidermis, pigment cells and lepidotrichia (Poss *et al.*, 2000a; Smith *et al.*, 2008). Lepidotrichia are elongated bony rays of dermal origin that run from proximal to distal end of the caudal fin to support the fin structure (Akimenko *et al.*, 2003). Each lepidotrichium is composed of concave and opposed hemirays with intra-ray mesenchymal tissue (Montes *et al.*, 1982). The caudal fin skeletal tissue is laid down by scleroblasts, skeletogenic cells equivalent to mammalian osteoblasts that secrete the lepidotrichia matrix (Hall, 2005). It has been reported that upon amputation of the adult zebrafish caudal fin, bony ray regeneration arises from the intra-ray mesenchymal cells that become disorganized, change their shape, re-enter the cell cycle and migrate distally (Poleo *et al.*, 2001; Nechiporuk and Keating, 2002;

Santos-Ruiz *et al.*, 2002). These anatomical features will serve as a standard reference for understanding the normal as well as deviant development hereafter observe in the regenerating tail fin of fish.

Initially a dose range study was conducted to select a dose that inhibits the BMP signaling effectively and also to validate its stage specific influence on the tail fin regeneration in *P. latipinna*. Following our observations on the effects of various dosages, 2.5mg/kg body weight was selected for further studies as appropriate in blocking the BMP signaling since lower dose did not cause any change and high dose was fatal to animals. Next, a morphometric study was carried out by amputating one third of the caudal fin, from the distal end, experimentally under hypothermic condition. Animals were randomized into two groups of eight animals each. The control group received injections of 1% dimethyl sulfoxide (DMSO), while treatment group received injections of LDN193189 (prepared freshly in 1% DMSO) at 2.5 mg/kg body weight. Drug was administered intraperitoneally and animals were examined on achieving a pre-decided stage for changes in morphology and regenerative outgrowth during the course of regeneration. The progression of fin regenerates of LDN193189 treated fish were compared with DMSO control fish.

The above study resulted in morphological changes in LDN193189 treated fishes when compared with the control group fishes. During the early stage, subtle but definite alterations were noticed. Blastema stage was delayed and later, differentiation stage showed structural abnormalities in caudal fin. Altogether it can be deduced that each stage was delayed. This data was also supported by the results of morphometric analysis of caudal fin regeneration, in which, the length of the regenerating fin was measured at the destined stage using digital vernier caliper. Regenerating fin was measured from the level of amputation to the distal end of the regenerate. For statistical analysis, the morphometric data was subjected to Bartlett test for homogeneity and the significance level of the treatment groups with control group was evaluated through Student's 't' test with 95% confidence limit. For multiple group comparison and difference between the groups the data was subjected to One Way Analysis of Variance (ANOVA) followed by Bonferoni's multiple range test using SPSS 20, statistical analysis software. A 'p' value of 0.05 or less was accepted as being statistically significant.

Necessity of BMP signaling during regeneration has been reported by Oumi and others (2012). Significant delay in wound closure, blastema formation and in early differentiation or growth stage was recorded in LDN193189 treated group animals. The blastema is enriched with differentiation and pattern forming signals that are known to be involved in bone tissue specification, such as FGFs, BMPs and Shh (Laforest *et al*., 1998; Poss *et al*., 2000b; Quint *et al*., 2002; Smith *et al*., 2008), which are secreted by the basal layer of the epidermis. It was also observed that during differentiation stage, length of the regenerate significantly declined ($p \leq 0.001$) in treated group.

In order to supplement the above morphological anomalies, histological profile of the regenerating tail was studied following standard methods. A smooth covering of cells was seen over the distal end of each lepidotrichium. Melanocytes and other mesenchymal cells were also noticed. Whereas these features were distinctly visible in control group animals, they were lagging far behind in development in the treated fins. Treatment group fins lacked a properly covered amputation wound, as can be understood from the absence of multi-layered epidermis, conjunctive tissue and pigment cells. From the initial observations it could be presumed that inhibition of BMP signaling hampers the formation of a functional wound epithelium and hence, retards the progression of regenerative response in the LDN193189 treated caudal fins. Similarly the blastema stage histological profile revealed a less keratinized apical ectodermal cap with trabecular spaces and was poorly formed which indicated functional impairment. Hence, only limited recruitment of blastemal cells were observed in LDN193189 treated group of animals. This could be due to the effect of LDN193189 on epithelial-mesenchymal interaction, which is mandatory for the blastema formation (Zhu *et al*., 2012). Moreover, blastema formation is the hallmark for epimorphic regeneration phenomena in nature, and is the first recognizable lead in the regenerative scenarios characteristic of the re-activation of embryonic genes recapitulating the events that occurred during embryonic development (Tsonis, 2008; Yokoyama, 2008).

During the early growth phase i.e. 5 days post-amputation (dpa) histological profile exhibited significantly delayed regeneration, disorganized and unusual pattern in regenerative growth. Additionally bending of actinotrichia was also observed in fins of the animals treated with LDN193189. Recent report has shown that BMP represents the first known signaling that induces actinotrichia formation in the regenerate (Thorimbert *et al*., 2015).

Further, at each stage of regeneration, we also confirmed whether intraperitoneal administration of LDN193189 successfully hampered BMP signaling in the fish fin. Two genes downstream of the BMP signal were selected, dlx5 and runx2, and their expression was checked in treated and control fins at each stage *viz.*, wound epithelium, blastema and early differentiation using real-time RT-PCR. Validity of the treatment method was tested 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 24hpa, 60hpa and 5dpa, confirming the inhibition of BMP signal.

There are ample evidences to believe that BMPs play important roles at multiple stages of endochondral bone formation and play critical roles at various stages of skeletal development (Hogan, 1996c; Wozney, 1998). BMPs and their receptors are present in chondrocytes and the adjacent perichondrium (Baur *et al*., 2000; Yi *et al*., 2000) and seem to affect the differentiation of chondrocytes. Studies using cell culture systems have shown that BMPs generally stimulate hypertrophic differentiation of chondrocytes (Shukunami *et al*., 1998; Boskey *et al*., 2002). BMP signaling is known to regulate bone as well as cartilage formation through discrete cascades and BMP-2 along with BMP-7 are reported to show osteogenic effects (Boskey *et al*., 2002). It was therefore thought pertinent to study the effect of BMP signaling on skeletal patterning during the early differentiation stage of regeneration.

In order to test the above notion tail fins were extracted on 5dpa and stained with Alcian blue and Alizarin red to identify the structural abnormalities, if any, in the cartilage/bone of the regenerate. As expected the result revealed many anomalies in LDN193189 treated animals and the most striking being the bent actinotrichia along the digits during later growth phase. Distal margins and intra-ray tissue were not properly sculpted when compared with control. Actinotrichia also lacked proper bifurcations at the distal end. Along with this BMP signaling inhibition also promoted lepidotrichia mineralization. Moreover, Alizarin red positive regions appeared only in proximal part of the fin and Alcian blue-positive chondrogenic regions appeared in the distal fin region which also suggests the presence of cartilaginous precursor cells in the ray.

In order to understand the molecular minutiae behind the observed structural anomalies, we evaluated the downstream targets of BMP signaling. The results of real-time RT-PCR vividly expressed significant down regulation of dlx5, runx2 and col10a1. It has been documented that dlx5 and runx2 are induced by BMP signaling in chick primary calvaria cells grown *in vitro* (Gersbach *et al.*, 2004). It is also known that dlx5 drives runx2 expression and the latter activates col10a1 resulting in osteogenic differentiation in mesenchymal cells (Holleville *et al.*, 2007). Besides, Osteonectin (Osn) and Osteocalcin (Osc) gene expressions were also declined notably. Osn and Osc are late differentiation markers involved in bone mineralization (Sousa, 2011). Altogether, our results suggests that during early differentiation stage there is delay in bone differentiation in treated fins due to subdued expression of molecular cues for osteogenesis as a consequence of BMP signaling inhibition.

During regeneration, the extracellular matrix (ECM) degradation is an early response after amputation, which allows migration of differentiated cells to the wound site where they undergo dedifferentiation (Brockes and Kumar, 2008). The ECM serves diverse functions and is a major component of the cellular microenvironment. The ECM components are deposited, degraded and modified because of which the ECM is considered as dynamic in nature and constantly undergoes a remodeling process. Collagen fibers, being one of the major components of the ECM, can profoundly influence the directed migration of cells, possibly by potentiating growth factor receptor signaling or by mechanically reinforcing cell migration (Hynes, 2009; Egeblad *et al.*, 2010). Collagen is a key element of the scar tissue, is an inhibitor of limb regeneration, indicating the suppressive role of collagen accumulation in tissue during regeneration (Satoh *et al.*, 2012). The major source of collagen in scar-forming wound healing is activated fibroblasts and myofibroblasts, which are contractile cells causing wound contraction, a process seen in scar forming wound healing but not in scarless regeneration. Blocking wound contraction and fibroblast accumulation at wound sites by degradable scaffolds with molecules such as integrin ligands can promote regeneration in skin and nerves in adult mammals (Yannas, 2005; Yannas, 2013). Activation of fibroblasts and secretion of collagen requires TGF-β signaling (Wynn, 2008; Zeisberg and Kalluri, 2013). Blockage of TGF-β signaling can dissolve fibrotic tissue and restore normal regeneration. Activation of BMPs, which counteract TGF-β signaling, can promote regeneration (Sugimoto *et al.*, 2012). Measurement of hydroxyproline is an efficient index of collagen deposition, since collagen contains significant amount of hydroxyproline amino acid in constant proportions. Therefore, to assess the role of BMPs on collagen synthesis during epimorphic regeneration the hydroxyproline assay was performed as described by Edwards and O'Brien (1980).

No significant difference in control and treated group was recorded after analysis of the collagen data during wound healing stage. Mean caudal regenerate collagen content in both groups remained same. Nevertheless, mean collagen content was found significantly declined during blastema and differentiation stage in LDN193189 treated group. Further, quantitative real-time PCR was also performed to reaffirm the above results at transcriptional level and found that col10a1 mRNA expression levels decreased significantly. Hence, the results matched with the results of biochemical and histological analysis substantiating the present notion of BMP signal regulating collagen turnover during epimorphosis.

ECM remodeling appears to be an essential requirement for normal appendage regeneration. The matrix reorganization at the site of amputation is crucial for the rapid migration of wound epithelium thus to achieve 'super healing'. In order to accomplish this process, ECM components have to be reorganized at the site of wound which is facilitated by matrix metalloproteinases (MMPs). MMPs function as key regulators of cell-ECM interactions during development. These enzymes are encoded by different genes and are implicated in several normal and pathological tissue remodeling processes such as wound healing, angiogenesis and tumor invasion (Forget *et al.*, 1999). Upregulation of MMPs is observed at early stages of regeneration in many regeneration competent organs and tissues, such as hydra, drosophila imaginal discs, amphibian limbs, and zebrafish fins (Bai *et al.*, 2005). Out of all members of gelatinases, MMP-2 as well as MMP-9 are responsible for one of the most fundamental and important processes in regeneration. Since these enzymes are thought to contribute largely during the very initial stages of growth and repair, a zymographic analysis was carried out during all the three regenerative stages to study the effect of BMP signaling on their activities.

Degradation of ECM also plays an important role in cell migration towards the underlying mesenchymal layer to form blastema. 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). Outcomes from the analysis of zymogram during wound healing stage reflected a sharp decline in the activities of the gelatinases in response to LDN193189 treatment at wound healing stage. 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 quantitative analysis clearly established that the suppression of expression of MMPs is occurring at the transcriptional level. Therefore, the current 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 mmp3 and mmp13 in stem cells (Otto *et al.*, 2007), the role of BMPs in regulating mmp9 was also subsequently brought to light in breast cancer cells (Shon *et al.*, 2009). However, Shon and coworkers (2009) found BMP-4 to be negatively regulating mmp9 expression, whereas the regenerating model system in the current study reveals a positive relation between BMP signaling and mmp9 expression.

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 the blastema stage in LDN 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 and blastema stage in LDN treated animals at transcriptional level. Therefore, it was revealed 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 for these genes to get upregulated. In order to reaffirm the temporal variation in MMP activity during regeneration, the major target of MMP namely collagen was quantified by estimating hydroxyproline in the regenerate. The collagen content was found significantly decreased in LDN treated group. Collagen is one of the molecular components of ECM. Unsolicited collagen turnover could be the reason behind the structural anomalies and delayed recruitment of blastemal cells observed in BMP signal inhibited animals.

Furthermore, epimorphic regeneration involves the generation of new stem cells, either by proliferation of the existing stem cells or by dedifferentiation of adult cells, which differentiate to form the lost appendage which is more or less similar in size and structure compared to the original lost structure (Brockes and Kumar, 2002). Just after amputation and wound healing, an initial bulge gives rise to a regeneration blastema, in which proliferating cells rapidly rebuild the fin. By now it is apparent that the major signal regulating tail regeneration is bone morphogenetic protein, and as reported, in tandem with Notch signaling (Beck *et al.,* 2003). Both the signals are required for cell proliferation although BMP pathway is independent of Notch signaling (Stoick-Cooper *et al.,* 2007b). Increase in cell proliferation in the regenerate is essential so that the regenerate can step into its successive stages without any hindrance. Decreased fin length observed in the receptor inhibitor-treated fishes may perhaps be the result of decreased cell proliferation.

Therefore, in this context cell proliferation studies were undertaken during blastema stage by *in vivo* BrdU labeling. The key principle of this method is that BrdU incorporated as a thymidine analog into nuclear DNA represents a label that can be tracked using antibody probes (Brockes and Kumar, 2002). Initially minimal BrdU was localized on the wound epithelium, as this is the phase that mainly depends on cell migration rather than cell proliferation. The blastemal phase is known to be composed of a mass of proliferating cells and therefore, as expected showed an abundance of BrdU labeling in the fins at this phase of regeneration in control fins. Conversely, cell proliferation activity was found significantly decreased, as revealed by less number of BrdU positive cells, in LDN treated animals. From the results it could be inferred that the blockage of BMPR by a specific inhibitor resulted in less number of cells entering the S-phase of the cell cycle and hence, more number of cells remained in the quiescent state compared to the controls. BMP signaling is known to be required for blastemal cell 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 fgf8 expression and proliferation of cells in the epidermis as well as the blastema. Moreover, inhibition of BMP activity using the BMP antagonist, noggin, caused a reduction in blastema cell proliferation and a reduction in bone matrix deposition. Similarly, in experiments using transgenic *Xenopus* tadpole with increased noggin expression, both tail and limb regeneration were inhibited (Beck *et al.*, 2006). The current results are in accordance with the previous reports on cell proliferation activities during post embryonic development.

Since proliferation events are often associated and dependent on apoptosis, it is also one of the event which is accompanying the initial matrix remodeling and cell proliferation, both of which are equally important for the successful formation of blastema. In fact, apoptosis is required during early stages of tail regeneration in *Xenopus laevis* and it was reported that inhibition of Caspase-3 activity during these stages leads to inhibition of the regenerative process (Tseng *et al*., 2007). Moreover, apoptosis is involved in sculpting of growing tissue 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). The BMP pathway has been shown to play an important role in the establishment of the dorso-ventral axis during development in both vertebrates and invertebrates (reviewed by De Robertis and Kuroda, 2004; Yu *et al*., 2010).

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 is required for successful regeneration (Makane, 2014). Thus, to understand, how BMP signaling influences apoptosis in regenerating fin, whole-mount immunohistochemistry of the fin for active Caspase-3 was performed. Caspase-3 protein is a member of the cysteine-aspartic acid protease family which plays a central role in the execution-phase of the apoptosis by its sequential activation (Alnemri *et al*., 1996).

At 24hpa, bright-field images exhibited activated Caspase-3enzyme expression in the wound epithelium in the fins of both control and LDN193189 treated animals. Apoptotic cells were seen in the entire regenerated fin. On the other hand LDN193189 treated fin showed increased activated Caspase-3 activity which was confined towards the wound epithelium and was also found to be more intense in relation to control fins. Apoptosis increased markedly after 60hpa in the fins of treated group animals. However, the pattern uniformity of Caspase-3 enzyme 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 inter-ray in LDN193189 treated group animals while the activity was uniform in the fins of control group animals.

Thereby, it is suggested that BMPR inhibition also promotes apoptosis during early stages. Programmed cell death is known to be involved in the sculpting of growing tissues in different development systems (Gilbert, 2000). During the growth phase Caspase-3 activity increased progressively by several times towards the distal region including actinotrichia, inter-ray and epidermis in the fins of LDN193189 treated animals compared to the fins of the control group animals where apoptotic activity was limited to the epidermis. Hence, there is a possibility that the morphological alterations observed during earlier stages of caudal fin regeneration may be a secondary consequence of general retardation caused by extensive apoptosis during regeneration in LDN193189 treated fins. Thus, it suggests that BMPR inhibition also promotes apoptosis during all stages of appendage regeneration in *Poecilia latipinna*. Hence, it supports the conventional notion that even though the massive tissue proliferation is required to rebuild the fin, an accompanying apoptotic event is quintessential for successful sculpting of the regrowing tissues.

In the light of all the above results, it could be deduced that BMP signaling is an essential component among the repertoire of molecular signals directing the process of regeneration in teleost fish. It is hoped that these results and the conclusions thereof will contribute, in some way, to our understanding of the mechanism of epimorphic regeneration. Although the cellular and molecular processes affecting the entire process are surely far from simple, a step by step identification and elucidation of the signaling pathways involved will eventually lead us to better understand, in detail, these processes.