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Temporal Expression Pattern of Peptides in the Regenerating Caudal Fin of Teleost Fish *Poecilia latipinna* With Special Emphasis on *krt15* and *myl-1*

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Abstract

Amongst several vertebrates, caudal fin of teleost fish has emerged as an excellent model to understand the mechanism of regeneration. By now it is well perceived that proper coordination of different regulatory signals are needed to facilitate the progression of successive stages of regeneration. Therefore, in the current study protein expression profile for caudal fin regeneration in *Poecilia latipnna* was brought into the focus. The pattern of protein turnover and variation in the expression level were observed across the stages of regeneration. Data from two-dimensional gel electrophoresis revealed the differential expression of proteins for wound epithelium, blastema and differentiation stages when compared to resting stage. Based on the computational analysis, of the peptide expression profile and subsequent sequencing, two peptides namely Keratin type I cytoskeletal 15 and Myosin light chain-1 were studied further at transcript level. *Keratin-15* was found to be up regulated at wound epithelium stage while its expression waned significantly in the blastema and differentiation stages of regeneration indicating its role in the formation of a functional wound epithelium. Western blot analysis of *Keratin-15* also concurred with transcript levels of *krt15*. However, *myl-1* transcript levels was observed to be dispensable for initial phase of regeneration whereas its elevated level in differentiation stage at both mRNA and protein level marks its noteworthy role in achieving the structural integrity of a regenerating caudal fin. This is the first observation of the involvement of *Keratin-15* and Myosin light chain-1 in the regulation of regenerative process in *Poecilia latipinna*.

Keywords: Two-dimensional gel electrophoresis; Caudal fin regeneration; Teleost fish; P. *latipinna*; *Krt15* and *myl-1.*

Introduction

Epimorphic regeneration involves the de-differentiation of existing tissue and recruitment of proliferative cells to form the new structure to restore the lost appendage. Such type of appendage regeneration is observed in a number of vertebrates including lizard tail, salamander limb and tail [1-4], and zebrafish caudal fin [5-7]. Amongst the models of vertebrate appendage regeneration, teleost caudal fin has emerged as an extensively used model to study epimorphic regeneration. Most of the caudal fin regeneration studies have been carried out in zebrafish due to its accessibility, its fast and robust regeneration and its simple architecture [8,9]. However, sailfin molly, *Poecilia latipnna* (Lesueur, 1821) have been served as an alternative to zebrafish, being more adaptive to the oriental laboratory conditions as well as certain experimental advantages owing to its relatively large size.

Following amputation of caudal fin in zebrafish, an epithelial layer covers the wound which gets thickened later on. Following this, mesenchymal cells migrate near the amputation plane beneath the newly formed wound epithelium, accumulate and proliferate to form the blastema and finally differentiate to replace the lost structures [5,9,10-14]. Wound healing or wound epithelium formation is achieved within 12-24 hours after amputation and blastema formation is achieved in 48-60 hours post amputation. Finally, the blastemal cells differentiate and restore structures including blood vessels, bony rays, and connective tissue by 15-20 days post amputation [6,9,12,15]. This time course of caudal fin regeneration in *P. latipinna* differs to that of zebrafish which has been reported in this study for the first time. The aforementioned stages of epimorphic regeneration are governed by certain cellular events which are ultimately regulated at molecular level. For each process to occur in a spatio-temporal manner, a large scale of protein turnover takes place. During protein turnover, several peptides need to be synthesized newly as per the need of the tissue and many to be downregulated.

There are reports on proteomic profile in regeneration models to unravel the conserved and diverse mechanisms amongst them [16-19]. It includes the regeneration in axololtl [20,21], *Xenopus laevis* froglet limb-bud [22,23] and zebrafish caudal fin regeneration [24]. Herein, *in P. latipinna*, two-dimensional gel electrophoresis was used to find out the overall expression pattern of peptides across the stages of epimorphic regeneration. This was followed by peptide sequencing of

selected spots based on their differential expression pattern across the stages of regeneration.

The sequencing results yielded two peptides, *keratin-15* and myosin light chain-1. As the requirement of keratin to get the structural integrity of newly formed epithelium has been reported in many vertebrates during development, adulthood and regeneration [25,26], further analysis of this peptide at transcript level was attempted. For myosin light chain-1, the gene *myl-1*, encodes two transcript variants expressed in fast skeletal muscle of zebrafish embryo [27] as well as it was reported in the fast twitch fibres and its knockdown has been shown to disrupt myogenesis [28]. Therefore, it was inferred that myosin light chain-1 is important during later stages of regeneration. Hence, in the current study, *myl-1* transcript level was checked during the caudal fin regeneration in *P. latipinna*.

Material and Methods

Animals and maintenance

Sailfin molly, *Poecilia latipinna*, (Lesueur, 1821), of both the sexes of the same age with an average size of 4-5cm and weigh about 5g were purchased from a commercial supplier. They were acclimatized and maintained in glass aquariums containing dechlorinated water with constant aeration, temperature range of 28-30˚C and photoperiod of 14:10 hours of light to dark cycle were maintained. The experimental protocols used in the study were carried out in accordance to the ethical guideline of Drugs and Cosmetics Rules, 1945 and was reviewed and approved by the Institutional Animal Ethics Committee (No. ZL/IAEC/15-2010) prior to the commencement of experiments.

Morphological observations and Tissue collection

One-third part of the caudal fins from all the animals was amputated in hypothermic conditions and allowed to regenerate. Fishes were observed under light microscope (Leica, USA) and achievement of each stage of regeneration was recorded at 25X magnification. Amputated fin tissues from 10 animals was collected and considered as a resting stage (R). Tissues for wound epithelium (WE) stage were collected at 24hpa. For blastema (BL) and differentiation (DF) stages, fins were collected at 60hpa and 5dpa respectively (n=10 animals per group for each experiment).

Two-dimensional gel electrophoresis

Fin tissues for all the stages, R (0hpa), WE (24hpa), BL (60hpa) and DF stage (5dpa) were collected and 10% homogenate was prepared using 2D lysis buffer containing (3-cholamidopropyl) dimethylamino-1-propane sulfonate (CHAPS), dithiothreitol (DTT) and protease inhibitor. Supernatant was collected the protein was precipitated by using cholorofrom-methanol precipitation method. The precipitated protein was solubilised in the sample buffer and the protein content was estimated by using Bradford method [29]. Absorbance was measured at 595nm with an ELISA plate reader (Metertech Σ960).

Thereafter, 40µg of each sample was subjected to first-dimensional gel electrophoresis with a 2D marker (Biorad, USA). Proteins were allowed to separate on the basis of their pI in the first-dimensional gel electrophoresis called isoelectric focusing with broad range immobilized pH gradient strip (IPG) 3-10 (Biorad, USA) followed by equilibration with DTT and Iodoacetamide (IAA). The strips were placed on to the resolving gel of SDS-PAGE and the proteins were separated further by electrophoresis. Gels were fixed with methanol:glacial acetic acid solution, stained with silver staining kit (Thermo Fisher, USA). Gel pictures were taken and analyzed using Biorad PDQuest version 8 analysis software.

Real-time PCR

All the tissues were collected in the TRIzol reagent for each stage. RNA isolation was achieved using the TRIzol method, and their purity was checked by the ratio of absorbance at 260 nm, and 280 nm and quality of RNA was ascertained with 1% agarose gel electrophoresis. 1µg DNase treated RNA was subjected to reverse transcription with cDNA synthesis kit (Applied Biosystems, USA). cDNAs were subjected to qRT-PCR analysis using select SYBR green mix (Applied Biosystems, USA) and specific primers. Primer sequences used for qRT-PCR are as follows: *krt15* Forward; 5'-GAGGCTGACATCAACGGTCT-3', *krt15* Reverse; 5'-TCCTGGGGCAGCATCCATTT-3', *myl-1* Forward; 5'-GCAACCCCAGCAATGAGGAA-3', *myl-1* Reverse; 5'-GAGTTCAGCACCCATGACTGT-3', *18SrRNA* Forward; 5'-GGCCGTTCTTAGTTGGTGGA-3' and *18SrRNA* Reverse; 5'-TCAATCTCGGGTGGCTGAAC-3'. Samples were measured in triplicate with *18SrRNA* as endogenous control. The fold change values were calculated using Livak and Schmittgen method [30].

Western blot

Equal amount of proteins were loaded on to 10% SDS-PAGE and electrophoresed at 100V for 2-3 hours. Proteins were transferred onto nitrocellulose membrane with 0.45 µ pore size at 100V for 100 minutes with transfer buffer containing methanol-tris-glycine. Membrane was developed for the presence of band for *Keratin-15* and *MYL1* using Anti-Cytokeratin 15 antibody Rabbit IgG (1:200, EPR1614Y, Abcam) and anti-MYL1 Rabbit IgG (Sigma-aldrich, USA) and anti-β-actin mouse IgG.

Statistical analysis

The data were subjected to One Way Analysis of Variance (ANOVA) followed by Bonferroni's multiple range tests using GraphPad Prism 5.0, statistical analysis package. The values are expressed as mean ± SEM. A 'p' value of 0.05 or less was accepted as being statistically significant.

Results

Morphology

To depict the process of caudal fin regeneration in *Poecilia latipinna*, gross morphological observations were made using light microscope. In **Figure 1A**, uncut fin can be observed along

with the marked amputation site. Following amputation, cut fin is seen in **Figure 1B**. By 24hpa, wound is covered with Apical Epithelial Cap (AEC) formed by epithelial cells which is shown in **Figure 1C**. Following wound epithelium stage, a bulgy structure called blastema at the end of each ray at 60hpa can be observed in **Figure 1D**. At 5dpa, newly formed lepidotrichia proximal to the amputation plane is visible which mark the onset of differentiation stage (**Figure 1E**).

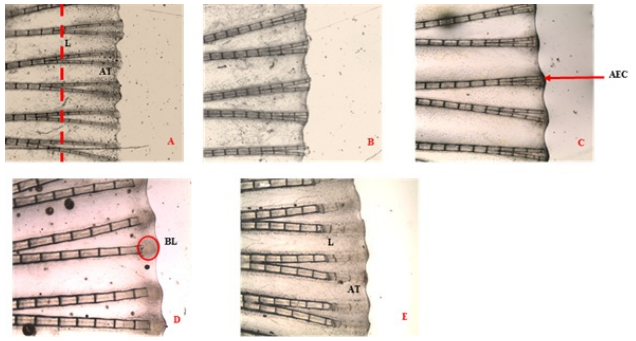

Figure 1: Stages of epimorphic regeneration in *Poecilia latipinna*: A. Resting caudal fin. Red dotted line represents amputation plane. B. Cut fin; Shows rough margin following amputation of fin. C. Wound healing (24hpa); An epithelial layer covering the wound (red arrow). D. Blastema (60hpa); A bludge (red circle) of proliferating cells beneath the AEC. E. Differentiation (5dpa); Newly formed lepidotrichia distal to amputation plane and actinotrichia formation towards the distal region of regenerating fin. n=10, Figure A, B, C the magnification was 25X and for figure D and E the magnification was 40X. L- Lepidotrichia, AT- Actinotrichia, AEC- Apical Epithelial Cap, BL- Blastema.

Peptides profile

Computational analysis of 2D gel images of the specified stages of regeneration of teleost fish, *P. latipinna* caudal fin was achieved with PDQuest software and it revealed a number of spots differentially expressed. In the resting stage, a total number of 182 protein spots were present while in wound healing, blastema and regenerated stage total 196, 201 and 193 spots were recognized respectively (**Table 1, Figure 2**).

Table 1: Total number of peptides expressed during caudal fin regeneration in *P. latipinna* at resting, wound epithelium, blastema and differentiation stage.

Stages	Spots
Resting	182
Wound Epithelium	196
Blastema	201
Differentiation	193

Further, based on the analysis, two proteins namely *Keratin-15* (annotated with blue triangle - pI 8.45, MW 45kDa) and Myosin light chain-1 (annotated with red circle - pI 6.68, MW 17kDa) were selected for their comprehensive study on the expression pattern in different stages of regeneration **(Figure 2)**.

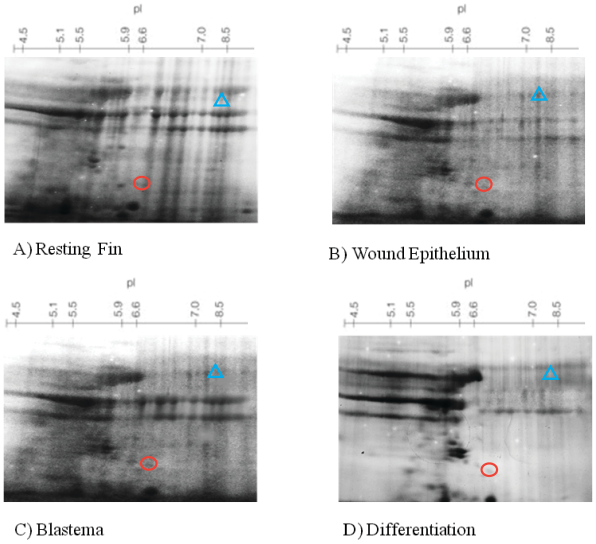

Figure 2: Representative two-dimensional gel images for all the stages of *P. latipinna* regenerating tail fin. Blue triangle represents spot with pI 8.45 and MW 45kDa for *Keratin-15*. Red circle annotates spot with pI 6.68 and MW 17kDa for MYL1. n=10.

Comparison of each stage with resting stage shared 83 numbers of spots in wound healing stage, 87 in Blastema stage and 92 in differentiation stage. **Figure 3A, 3C** and **3E** show the Venn diagram of the comparison made between resting stage with that of different stages of regeneration namely wound epithelium, blastema and differentiation stage. Total 40, 57 and 37 numbers of spots were observed up-regulated in wound healing, blastema and differentiation stage respectively and 40, 30 and 62 spots were down-regulated in the WE, BL and DF stage with respect to the resting stage (**Figure 3B, 3D and 3F**).

Transcript levels of *krt15* and *myl-1*

Transcript level analysis was carried out using quantitative real time reverse transcriptase PCR.

Table 2: keratin type 1 cytoskeletal 15 like and myl-1 light chain transcript fold change values during *P. latipinna* caudal fin regeneration. SEM= standard error of the mean. ***p ≤ 0.001, n=10 per group.

Stages of Regeneration	Fold change (Mean ± SEM)	
	krt15	myl-1
Wound Epithelium	2.34 ± 0.45***	0.0138 ± 0.00326***
Blastema	0.069 ± 0.0089***	0.186 ± 0.01241***
Differentiation	0.0146 ± 0.00254***	15.9959 ± 5.0580***

It is apparent from the analyzed results that Keratin type I cytoskeletal 15 showed a significant increase during wound epithelium stage (24hpa) with an average fold change value of

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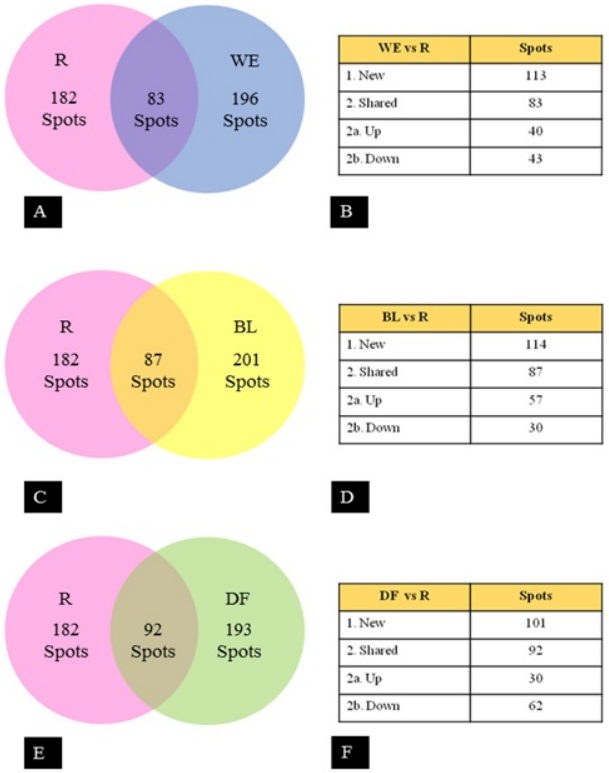
2.34 compared to the resting stage. However, the same was found to be significantly decreasing during the BL (60hpa) and differentiation stage (5dpa) with the mean values of 0.069 and 0.0146 respectively when compared to the resting stage (**Table 2, Figure 4**). On the other hand, myosin light chain-1 showed reduced level during 24hpa and 60hpa but its level increased significantly in the 5dpa (**Table 2, Figure 5**).



WE vs R	Spots
1. New	113
2. Shared	83
2a. Up	40
2b. Down	43

BL vs R	Spots
1. New	114
2. Shared	87
2a. Up	57
2b. Down	30

DF vs R	Spots
1. New	101
2. Shared	92
2a. Up	30
2b. Down	62

Figure 3: Venn diagram representing the overlapping spots in the gel when resting stage is compared with WH (A), BL (C) and DF (E) stages. The tables (B, D, F) shows the newly expressed proteins and from the shared proteins the ones that are up-regulated and the ones which are down-regulated. n=10. (R: resting stage, WE: wound epithelium stage: BL: Blastema stage; DF: Differentiation stage).

Protein expression profile for *Keratin-15* and *myl1*

To confirm our transcript level results, western blot was performed for *Keratin-15* and *myl-1* for all the stages for regeneration in caudal fin of *P. latipinna*. Protein levels of both, *Keratin-15* and *myl-1* did match with their mRNA expression levels. When compared to the resting stage, *Keratin-15* (45kDa) was found to be increased significantly during WE stage whereas it went down during BL and DF stages (**Table 3, Figure 6**). Quite contrary to *Keratin 15*, *myl-1* protein (17kDa) expression was found to be decreased during WE stage and its levels were increased noticeably during BL and DF stages.

Table 3: Densitometry analysis of western blot images for relative intensities of Keratin type 1 cytoskeletal 15 like and Myosin light chain-1 during *P. latipinna* caudal fin regeneration. SEM= standard error of the mean. ***p ≤ 0.001, n=10 per group.

Stages of Regeneration	Intensity (Mean ± SEM)		
	Keratin-15	myl-1	β-actin
Resting	52.15 ± 6.13	32.34 ± 3.82	84.24 ± 9.45
Wound Epithelium	89.44 ± 9.30***	9.12 ± 0.97***	85.69 ± 8.92
Blastema	38.55 ± 4.78***	13.32 ± 1.43***	87.58 ± 9.61
Differentiation	20.87 ± 3.33***	69.58 ± 7.12***	84.89 ± 9.87

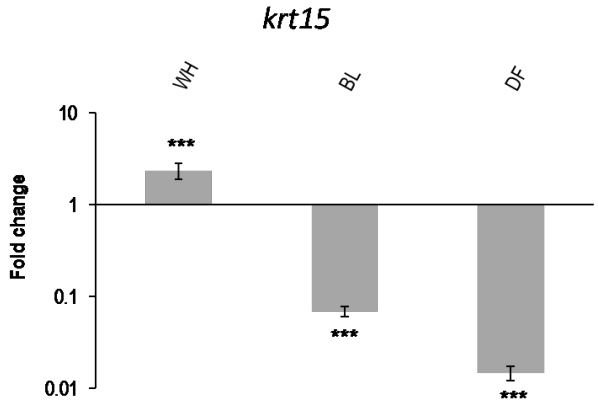


Figure 4: Keratin type 1 cytoskeletal 15 like (krt15) transcript fold change expression during P. latipinna tail fin regeneration. Error bars represent standard errors of the mean. ***p ≤ 0.001, n=10.

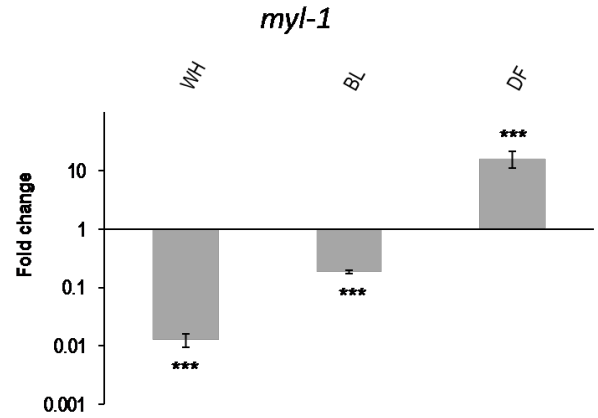


Figure 5: Myosin light chain-1(myl-1) transcript fold change expression during P. latipinna tail fin regeneration. Error bars represent standard errors of the mean. ***p ≤ 0.001, n=10.

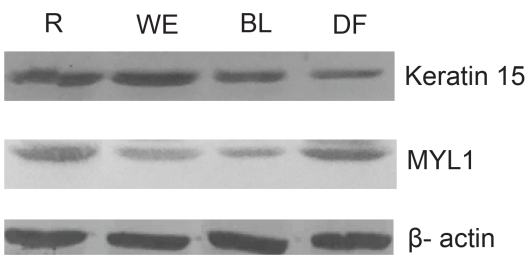


Figure 6: Western blot of *Keratin-15* and *myl-1* during the stages of regeneration in caudal fin of *P. latipinna*. β-actin was taken as loading control, n=10.

Discussion

Vertebrate appendage regeneration has attracted a huge attention in current times. As the regenerative capability decreases with increasing complexity in tissue organization, understanding the mechanism of regeneration has become inevitable. To understand the mechanism of regeneration, a number of model organisms are available in the field of regenerative biology. Teleost fish is one such model organism which has been documented extensively for studying regeneration process. In our study, we have explored caudal fin regeneration in teleost fish, *Poecilia latipinna*, as a model of epimorphic regeneration to illustrate the molecular mechanism with respect to peptides.

It is previously known that regeneration involves controlled expression of peptides and transcripts of signalling molecules at each stage of epimorphosis [18,19,31]. Therefore, to identify the stage-specific expression of peptides, we performed two dimensional gel electrophoresis. When the fin tissues of the selected stages of regeneration were subjected to two-dimensional gel electrophoresis, some of the spots were found to be up-regulated and expressed in a stage-wise manner. In addition, few spots exhibited down regulation, and their presence was not observed at a specific time point. Among all the spots, few remained constantly present in all the stages stating their requirement for maintaining the homeostasis of the growing tissue.

During the peptide screening, two peptides, *keratin-15* and myosin light chain-1 were focused for further studies based on their differential expression level across the stages of regeneration. *Keratin-15* is a type of soft alpha keratins. *Keratin-15* is present in the epithelial cells and considered as epidermal stem cells marker [32]. Keratins are important constituents of intermediate filaments of the intracytoplasmic cytoskeleton of epithelial cells. Role of keratin proteins was reported during wound healing and in several healing responses [33-35]. It has also been observed that various keratins are expressed at high levels in regenerating tissues [36-40]. Imboden et al. [41] identified cytokeratin 8 as a suitable epidermal marker during zebrafish caudal fin regeneration. In the regenerating axolotl limb study, regulated expression of KRT5 was observed during blastema which was suppressed in the basal layer of the AEC. Also, KRT5 and KRT 17 were reported as useful marker gene for AEC formation in the limb [42]. Knockout study of keratin 17 in mice showed delayed wound healing [43] and its reintroduction leads to improved healing. During in-vitro study, it was suggested that keratin plays an important role in improving the speed of wound healing [44]. Moreover, keratin formulation can also improve the epidermal migration by the upregulation of keratin gene expression [45,46]. Few of the mammalian type keratin markers such as keratins 6, 16, and 17 have also been identified in the lizard [47-49]. Therefore, herein the expression of keratins, suggests the role of keratins for the successive formation of the epithelial layer. The possibility of keratin involvement in the formation of epithelial covering has remained high during the development of regeneration. Therefore, the study of cytoskeletal keratin 15 was undertaken further at transcript level expression during regeneration in *P. latipinna*. Elevated level of krt15 transcript was found at WE stage, which again confirms the role of keratin 15 in forming wound epithelium. However, expression of the same gene went down in blastema and differentiation stage. Nonetheless, western blot analysis of Keratin 15 coincided with transcript levels of *krt15* and hence strengthens its requirement during WE stage in caudal fin regeneration in *P. latipinna*.

Other than keratin 15, our study revealed a significant change in expression of myosin light chain-1 upon peptide sequencing. *myl-1* encodes two transcript variant of myosin light chain 1/3 skeletal isoform, a small polypeptide alkali light chain and functional unit of myosin, expressed in fast skeletal muscle [27]. Along with Myosin heavy chain, myosin light chain forms a functional unit for smooth muscle contractility and migration and proliferation [50].

During the proteomic study of axolotl regenerating limb it was noted that about one-third of the cytoskeletal proteins were downregulated like sarcomeric proteins of skeletal muscle such as TNNT3A, TM7, myosin light chain 3 (MYL3) and MYL5 [21]. Kelly and Buckingham [51] have reported that myl-1 expressed in mouse fast-twitch fibres. Burguie`re and his colleagues [28] had reported myl-1 as earliest expressed marker in the fast-twitch precursor cells in the zebrafish embryo, and its knockdown disrupts myogenesis. In *P. latipinna*, results revealed down regulation of the myl-1 gene at the wound healing and blastema stage while in the differentiation stage it was found to be increased at both transcript as well as protein level suggesting the involvement of myosin light chain 1/3 during muscle contractility for the movement of the fish, *P. latipinna*. Hence, the myl-1 expression during differentiation phase of regeneration points towards the active involvement myosin light chain 1/3 regulatory protein in regaining the cytoskeletal integrity.

In brief, our study bring to fore for the first time a peptide profile for caudal fin regeneration in *P. latipinna*. Further, based on the differential expression pattern, two proteins namely *Keratin-15* and Myosin light chain-1 were sequenced and their expression pattern during different stages of regeneration was reaffirmed through both transcript as well as protein level analysis.

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