GENERAL CONSIDERATIONS

Upon damage or loss of tissue, all organisms, across the evolutionary hierarchy, react by evoking an organised biological response to repair the damage in order to improve their chance for survival. However, the extent of restoration of the lost structure and function always remains unequal among organisms. In fact, variation of the response towards damage, among a group of organisms and in the same individual among different organs has also been documented (Alibardi, 2017). This enormous process of repair and restoration of the lost or defunct tissue or organs is known as regeneration. In humans, hematopoetic cells in blood, epidermal cells of epithelia, hepatocytes of liver, etc. show regenerative ability but other parts like the heart, spinal cord and limbs lack this ability to regenerate (Birbrair *et al*., 2013). In infants, fingertip regeneration (McKim, 1932) and rib regeneration (Philips *et al*., 2005) has been observed, which is missing in the adult humans. Several other higher vertebrates including aves and mammals also show limited regenerative ability. Even with this ability, they do not match the response generated by the lower vertebrates which show enormous capacity to regenerate tissues across their body. Since appreciable regenerative ability has been reported from the members of cnidaria to reptilia, several studies on regeneration have classically involved the use of models like hydra, planaria, zebrafish, axolotl, newt and lizard to understand the mechanisms of regeneration (Brockes and Kumar, 2002; Akimenko *et al*., 2003; Agata *et al.,* 2007; Alibardi, 2010; McLean and Vickaryous, 2011).

Loss of any organ from the body disrupts, in at least some way, the biological system of the animal and its systemic functioning. Even during many diseases, loss of tissue eventually leads to death, if not disability. To improve all the conditions, proper treatment should be provided to the organism. Similarly, the process of regeneration can be induced in the localised cells or tissues and development of a new structure can be achieved. Thus, gaining an understanding of regenerative processes can answer the questions raised regarding mechanism of repair and also about the possibility of its extrapolation on to humans. Considering the regenerative medicine

approach, if we are able to mimic the conditions that prevail in the tissues during regeneration of organs in animal models, we are likely to succeed in regenerating lost tissues in humans. Hence, researchers are in search of all the possible ways which can induce a response to restore the loss. An encouraging observation is that on an evolutionary scale, research indicates significant conservation of genes and proteins among mammals and the lower vertebrates (Vitulo *et al*., 2017). Therefore, understanding the mechanism of regeneration in lower vertebrates can very well help us move a step closer to unearth the reasons due to which mammals fail to regenerate like their less evolved counterparts (Mathew *et al*., 2006).

Regeneration can be classified into two categories based on the cellular mechanism involved. In first, morphallaxis involves remodelling of the existing tissue to restore the lost body part. Stem cells are involved during the process and little new growth is observed. Such ability is found in hydra (Bosch, 1998). Second is epimorphosis or epimorphic regeneration, which involves the dedifferentiation or transdifferentiation of the adult structure or/and proliferation of the stem cells to form a new structure. This type of regeneration is observed in crustaceans, molluscs, teleost fishes, urodele amphibians, lizards and even in some mammals (Flores *et al*., 1992; Hopkins, 1993; Uchida *et al*., 2000; Nye *et al*., 2003; Poss *et al*., 2003; Clause and Capaldi, 2006; Han *et al*., 2008).

Epimorphic regeneration is further subcategorized into two types. In the first type, direct transdifferentiation or limited dedifferentiation and/or proliferation of the stem cells has been observed while in the second type, a pool of undifferentiated cells proliferates and forms a cone shape structure called blastema which further elongates and re-patterns to restore the lost structure. The process of epimorphosis also involves several events like dedifferentiation of post-mitotic cells, activation of multipotent progenitor cells, cell proliferation, pattern formation and in some cases, transdifferentiation of specialized cells to regain the lost body parts (Poss *et al*., 2002; Tanaka, 2003; Brockes and Kumar 2005; Alvorado and Tsonis, 2006). Three distinct stages of epimorphic regeneration are (i) wound healing via wound epithelium formation; (ii) formation of a regeneration blastema, a population of mesenchymal

undifferentiated progenitor cells that is responsible for proliferation and (iii) regenerative outgrowth associated with morphogenesis (Poss *et al*., 2003).

To understand the molecular and cellular changes which occur during epimorphic regeneration, various studies have been carried out in zebrafish, axolotl and newt as they have the natural ability to regenerate. During several events like inflammation, matrix digestion, matrix reorganization, wound healing, programmed cell death, proliferation, differentiation and tissue patterning, various molecules have been identified to be playing important role and their alteration has led to impaired growth or inhibition of the regenerative process. After amputation or loss of an organ, wound closure occurs within few hours and the amputation site is covered due to migration of the existing epithelial cells and wound epithelium (WE) is formed (Call and Tsonis, 2005). Few of the genes identified during early stages of regeneration are *mmp9* and *msx2*, which are independently expressed prior to wound closure. Among the many such genes, the MMPs (matrix metalloproteinases) are responsible for matrix digestion and remodelling, contributing significantly to the formation of a functional, multi-layered wound epithelium called the apical epithelial cap (AEC) (Call and Tsonis, 2005; Vinarsky *et al*., 2005). The specialized structure, so formed, is equivalent to the apical ectodermal ridge (AER) of the developing limb bud in amniotes (Summerbell, 1974; Saunder *et al*., 1976; Saunders, 1998).

Beneath the epidermis, a mass of cells accumulates and forms a cone shaped structure at the distal tip of the appendage, which is either constructed from mature residing tissues or residential stem cells activated to form blastema (Agata *et al*., 2007). Blastema formation depends on the signals derived from nerves or at least requires presence of nerves (Tsonis, 1996). Expression of HoxA9 and HoxA13 during formation of the distal stump of the amputated limb was reported in axolotl (Gardiner *et al.*, 1995). Several neurotrophic factors generated from the neurons stimulate cell proliferation (Mescher and Tassava, 1975; Maden, 1978; Mescher, 1996; Nye *et al*., 2003). Blastemal cell proliferating factors like FGFs and Dlx3, are found down-regulated due to the loss of nerves (Mullen *et al*., 1996; Cannata *et al*., 2001; Christensen *et al*., 2001). Several other neural factors like iron binding protein transferrin, GGF (neuregulin) and fibroblast growth factor 2 have been identified to be

contributing as well (Mescher and Munaim, 1984; Mullen *et al*., 1996; Wang *et al*., 2000).

Further, blastemal cells proliferate and differentiation stage initiates in the regenerating appendage. For the proximo-distal and dorso-ventral patterning during axolotl limb regeneration, retinoic acid has been identified to be playing an important role (Ludolph *et al*., 1990). It acts on cell surface molecule Prod1 (proximodistal-1), a newt CD59 homolog, which is an important factor for developing salamander limb blastema, defining its proximo-distal identity (da Silva *et al*., 2002).

During epimorphic regeneration study, signalling pathways of the cellular processes such as FGF, Wnt/β-catenin, RA, Shh, Activin and BMP have always remained the focus of research. A factor Lef1 (lymphoid enhancer-binding factor 1) of Wnt signalling pathway is required for zebrafish fin regeneration (Poss *et al*., 2000a). Further, Dkk-1 is an inhibitor of Wnt signalling pathway that blocks both Wnt and FGF signalling during limb regeneration (Kawakami *et al*., 2006). In 1976, Gospodarowicz had reported the role of FGF in wound healing, blastema formation and regenerative outgrowth. FGF signalling during blastema stage also activates *Shh* expression and its inhibition down regulates *msx* and *Shh* expression (Poss *et al.,* 2000b; Lee *et al.,* 2005; Thummel *et al.,* 2006a; Lee *et al.,* 2009). Other than RA signalling (White *et al.,* 1994; Geraudie *et al.*, 1995; Geraudie and Ferretti, 1997), Activin-βA is also involved (Jaźwińska *et al*., 2007) in patterning. Controlled expression of several molecules like *shh*, *ptch1* and *bmp2* is responsible for skeletal regeneration and patterning in zebrafish (Laforest *et al*., 1998). Also, their signalling inhibition alters the regenerative process (Quint *et al.,* 2002; Smith *et al.,* 2006)**.**

In the past few years, attempts to find out the role of several molecules at particular time point have been made with advanced tools. These advanced methods have allowed researchers to find out several other important molecules involved in the signalling. Changes in the epigenetic markers and in micro RNAs during newt lens regeneration have been reported (Maki *et al*., 2010; Nakamura *et al*., 2010). Hypermethylation of MFCS1 (mammals-fishes-conserved-sequence 1) and Shh enhancer, has been observed in *Xenopus* and moderately in axolotl and newts too

(Yakushiji *et al*., 2007). Studies have been carried out, wherein, three (*klf4, sox2* and *c-myc*) out of six transcriptional regulators have been identified to be functional in the proliferating blastema in newt lens regeneration (Maki *et al*., 2009). Lin28 has been identified as being upregulated in its expression during axolotl limb regeneration (Rao *et al*., 2009). Various proteomic studies of epimorphic regeneration include the study of axolotl regenerating limb by Rao and associates (2009), proteome profile of zebrafish caudal fin regeneration using 1D followed by LC-MS/MS and two-dimensional gel electrophoresis coupled with MALDI MS/MS (Singh *et al*., 2011) and in *Anolis carolinensis* tail of 72 hours post-amputation (Hutchins *et al*., 2014).

Additionally, new approaches to use molecular tools to address the problem of regeneration are rapidly emerging and attempts to find out important regulators during regeneration are being made in various models of regeneration including zebrafish, axolotl and urodeles. Till date, most of the research on naturally evolved epimorphic regeneration has focused on anamniotes including zebrafish and newts. Although explored in context of ecological costs and benefits, very less is known about the sequence of cellular and tissue level events of lizard tail regeneration or of any other reptilian group.

Epimorphic regeneration has been observed in reptiles. It can be considered as a model possessing intermediate regenerative ability between amphibians and mammals. Crustaceans, fish, amphibians, etc. possess higher regeneration ability, whereas higher vertebrates including birds and mammals possess restricted lower regenerative ability. Studies of reptilian group regeneration include crocodile tail, jaw and turtle shell regeneration (Bellairs and Bryant, 1985; Carlson, 2007). Regeneration has also been observed in living fossil *Sphenodon punctatus*, chelonians (turtles and tortoises) and crocodilians (crocodiles, alligators and caiman) (Bellairs and Bryant, 1985; Webb and Manolis, 1989; Carlson, 2007). Among the reptiles, lizards form the largest group in terms of species richness, which together with the snakes and amphisbaenids (Evans, 2003) possess regenerative ability in nerve cells, parts of lower mandibular axon, parts of the limb and tail, while skin recovery is reported in snakes (Simpson, 1961; Bryant, 1970; Bellairs and Bryant, 1985; Smith and Barker, 1988). Such studies on reptilian systems have been

neglected despite the fact that the lizard represents the best non-mammalian amniote group, to analyze molecular factors involved in the regeneration of various tissues of tail.

Moreover, lizards form the closest group of organisms in evolutionary hierarchy to mammals, sharing similar histological features, making them an attractive model to study regeneration. They possess pre-fracture planes, responsible for intervertebral autotomy observed in their tail. With this, lizards can shed their tail from the plane under certain conditions like grabbing, biting or scaring (Vitt 1983; Reichman 1984; Goss 1987; Maginnis, 2006). Also, for biological or morphological replacement to favour metabolic survival, speed of movement, prehensility, social behaviour etc, create a selective pressure for regeneration upon loss of a non-vital organ, the tail (Alibardi, 2010).

Lizards that have been used as amniote regeneration models include leopard gecko (McLean and Vickaryous, 2011), green anoles (Hutchins *et al.,* 2014) and northern house gecko. Yellow-bellied house lizard, *Hemidactylus flaviviridis* has been used in our department since many years now (Kumar and Pilo, 1994; Pilo and Suresh, 1994; Pilo and Kumar, 1995). Early studies include analysis of histological, biochemical and metabolic alterations during regeneration (Kumar and Pilo, 1994; Pilo and Suresh, 1994; Pilo and Kumar, 1995; Yadav *et al*., 2012). Subsequent studies on tail regeneration in *H. flaviviridis* revealed that few growth factors and neural peptides like FGFs and EGFs are essential for successful regeneration (Pilo and Suresh, 1994; Yadav *et al*., 2008; Suresh *et al*., 2010; Sharma *et al*., 2011; Pillai *et al.*, 2011; 2013; Yadav *et al.*, 2012).

Cellular and molecular alterations during tail regeneration were noted in *H. flaviviridis*. All the events need to progress in a proper coordination and controlled manner, which is always achieved by not only one or two peptides or molecules but several sequential expressions of peptides, collectively giving signals for the regeneration initiation and subsequent elongation followed by growth. Also, few molecules need to be down regulated as their function might naturally interfere in progress of regeneration by providing inhibitory cues.

Therefore, a hypothesis can be constructed from all the observations that there could be many more unidentified regulatory proteins working in unison to facilitate various cellular events of regeneration. Hence, a more detailed study on the overall expression profile was necessary to understand tail regeneration of *H. flaviviridis*.

Therefore, the overall aim of the present study was to evaluate the protein expression profile during various stages of tail regeneration in lizard *Hemidactylus flaviviridis.*

To achieve the above milestone, the work was intiated with the observation of overall expression profile of peptides during each stage of regeneration in the northern house gecko with two-dimensional gel electrophoresis (2D-GE). 2D-GE profile was not available for the regenerating tail of *H. flaviviridis* prior to this study (Chapter 3).

Two-dimensional gel electrophoresis is a very important proteomics tool, which generally appraises about the abundance and presence of proteins at a definite time point. 2D-PAGE can separate more than 1000 proteins in a single gel and separates protein mixture on the basis of the isoelectric point (pI) and molecular weight (MW) of peptides (Choe and Lee, 2000). Firstly, proteins separate according to their pI linearly in a pH gradient called as Isoelectric focusing (IEF). And the second dimensional separation can be achieved by using SDS-PAGE on the basis of their respective molecular weight.

From our previous studies, it was always observed that peptide expression varied among the stages of tail regeneration in northern house gecko (Pillai, 2012). Therefore, to resolve more peptides, the study was started with two-dimensional gel electrophoresis. 2D profile of the four-distinct stages: resting tail, wound epithelium, blastema and complete restoration/ regenerated stage. Results showed more than 200 spots in each stage and among them more than 50 spots showed change in their expression pattern across the stages of regeneration. Two differentially expressed spots were selected for the identification via nano-liquid chromatography/mass spectrometry (nano LC-MS/MS). It is liquid chromatography coupled with mass spectrometry, which measures the mass to charge ratio of charged particles

separated through the high-performance liquid chromatography. It is a well-established analytical technique used for separation, detection and identification of a molecule from a complex protein mixture.

One more approach was also considered in the study for protein profile which was label-free detection and quantification method. The major advantage of this method is that comparative quantification can be performed across many samples simultaneously (Wong and Cagney, 2010). When protein samples of all the four stages were subjected to Label-free detection, results provided both qualitative and quantitative data. Total 261 proteins were identified using SWISS-PROT and TrEMBL tools. Out of 261, 35 proteins were found to be expressed differentially for all four stages. Those differentially expressed proteins were categorized based on their involvement in particular cellular process, *viz*., apoptosis, inflammation, structural proteins, regulatory proteins and metabolic proteins. Bcl-2, an anti-apoptotic protein, was found to be up-regulated whereas BAD, a pro-apoptotic protein was down-regulated during WE and BL stages signifying a tight regulation between cell proliferation and apoptosis. Such controlled activity of pro-apoptotic as well as anti-apoptotic proteins during fin regeneration has been reported in zebrafish (Hasegawa *et al*., 2015). Obvious changes in the levels of inflammatory mediators were reported. Anti-inflammatory proteins, IL10 and platelet-derived growth factor receptor alpha precursor were found to be elevated during early stages of regeneration which is a pre-requisite for preventing scaring and promoting regeneration (Harty *et al*., 2003). Tumor necrosis factor, a pro-inflammatory mediator was found to be decreased during WE and BL stages displaying a balanced inflammation. Most of the regulatory protein and proteins involved in metabolism remained unchanged during regeneration and so was the case for structural proteins. From quantitative data obtained for structural proteins, significant up-regulation in the levels of Keratin type I cytoskeletal 15 (KRT 15) proteins was reported for WE but in BL it was decreased noticeably. Apart from this, Myosin light chain 1/3 protein also showed change in its expression pattern across the stages of regeneration. It was down-regulated during BL stage but was elevated during regenerated stage, presenting its requirement for gaining structural stability in newly formed tail. These same two proteins were obtained upon identification of selected spots from 2D gels via nano LC-MS/MS.

One of these two spots was identified as Keratin type I cytoskeletal 12/15 like peptide, which is highly expressed in the proliferative stages of regeneration where the wound epithelium has formed and blastemal cells are in the proliferative stage. This spot was absent in the resting as well as in the fully regenerated tail. Therefore, it was thought that it might play a significant role during wound epithelium and blastema but not in the differentiation process of regeneration. Keratin type I cytoskeletal 12/15 like, are important constituents of intermediate filaments of intra-cytoplasmic cytoskeleton of epithelial cells and of epithelial keratin type also called as soft alpha-keratins. Due to enormous role of keratins in the several biological events like development, adulthood, regeneration and hyperproliferation (Fuchs *et al*., 1987; Kallionen *et al*., 1995), it was further focused during the study. It was also documented that keratin is involved in epithelial cell growth and protein synthesis. Further, Keratin 1 lacking cells showed decreased protein synthesis along with decreased signalling of Akt pathway (Kim *et al.,* 2006). Elevated level of Keratins was also noted in various regenerating tissues (Smoller *et al*., 1989; Ferretti and Brockes, 1991; Tsonis *et al*., 1992; Ferretti *et al*., 1993; Martorana *et al*., 2001). Cytokeratin 8 was reported as epigenetic marker during zebrafish caudal fin regeneration (Imboden *et al*., 1997) and keratin 5 and keratin 17 as AEC marker in regenerating axolotl limbs (Moriyasu *et al*., 2012). Expression of keratins by these cells suggests the role of keratins for formation of epithelial layer following amputation of an appendage.

Although keratin is a structural protein, important for maintenance of skeletal framework of the cells or tissues, possibility of keratin involvement in the formation of epithelial covering has remained high during the study of regeneration. Knock out study of Keratin 17 in mice showed delayed wound healing (McGown *et al*., 2002). Also, their reintroduction leads to improved migration and cell growth. During *in-vitro* study too, it was suggested that Keratin is playing important role in improving speed of wound healing (Kim *et al.,* 2006). Furthermore, Keratin formulation can also improve the epidermal cell migration by up-regulation of keratin gene expression (Perez *et al.*, 2009; Pechter *et al.*, 2012).

Therefore, it was thought that the Keratin type I cytoskeletal 12/15 like proteins might be involved in the cellular changes and favouring wound healing in regeneration of *H. flaviviridis* tail. Because the peptide for keratin type I cytoskeletal 12/15 appeared during the wound epithelium and blastema stage, we were also interested to find out whether its gene expression is induced or not. Hence, its transcription level was checked among the regeneration stages. When spot identification was made following 2D-GE, *Anolis carolinensis* database was used as that of *H. flaviviridis* was unavailable*. A. carolinensis* belongs to the reptilian group hence its use for the comparison with *H. flaviviridis* is justified. The conserved region of identified peptide's nucleotide sequence was used for designing specific primers. And mRNA expression results so obtained showed the same expression pattern as that of the peptide. Detailed review of literature states that a krt isotype is involved in the corneal epithelium formation (Kao *et al.,* 1996; Irvine *et al*., 1997; Allen *et al*., 2016). Hence, the involvement of *krt15* in regenerating tail of *H. flaviviridis* was inferred for the formation of the wound epithelium.

Second spot of our interest was the one which was present in both, the normal tail and the regenerated tail but not during the wound epithelium and blastema stage. There are several proteins in the system which need to be down-regulated and their presence might affect the regeneration process. Also, upon loss of the organ, several functions of tail do not require their expression e.g. few proteins which are involved only in the mobility of the tail are not needed and their activation and expression is not required. The second selected spot was identified as Myosin light chain 1/3 skeletal isoform (MLC 1/3).

Myl-1/3, a small polypeptide alkali light chain which is the functional unit of myosin, expressed in fast skeletal muscle encoded by two transcript variants of gene *myl-1* (Periasamy *et al*., 1984) was investigated. The isoform diversity is greatly controlled at transcription level by cis-acting regulatory modules (Kelly and Buckingham, 2000). Kelly and Buckingham in 1997 have reported that Myosin light chain-1 is expressed in mouse fast-twitch fibres. In 2011, Burguiere and his colleagues reported *myl-1* as earliest expressed marker in the fast-twitch precursor cells in zebrafish embryo and also that its knockdown disrupts myogenesis. Hence, the MLC 1/3 expression during

the differentiation phase of lizard tail regeneration points towards the active involvement of this regulatory protein in the muscle differentiation, which is the predominant cellular activity taking place at that stage of regeneration. Its mRNA expression levels were also found at the regenerated stage while in the WE and BL stage, it was down-regulated.

Moreover, to understand the evolutionary conservation of Krt15 and MLC 1/3 skeletal isoform among vertebrates, a parallel study was conducted in regeneration model teleost fish, *Poecilia latipinna*. Among teleost, zebrafish is an excellent model of epimorphic regeneration. They exhibit regenerative capability in almost all parts of their body including scales, muscles, spinal cord and heart in addition to their fins (Akimenko *et al*., 2003; Poss *et al*., 2003). Their caudal fin containing bony rays (Montes *et al*., 1982; Becerra *et al*., 1983; Géraudie and Singer, 1992) can also be considered as homologous tissue of tail and limb of vertebrate (Hinchliffe, 2002). However, considering the ease and ready availability, the teleost fish sailfin molly, *Poecilia latipinna*, was used in the present study. Moreover, we have a lot of inhouse historical data about *P. latipinna* since we are using this model for quite some time. Additionally, *P. latipinna* is proved to have a better survival rate in our laboratory condition and their relatively large sized caudal fin (compared to zebrafish) is a boon, since less number of animals are to be sacrificed to obtain a specific amount of tissue to achieve each milestones of a study which is considered a good practice in accordance with the animal ethical guidelines.

Once the caudal fin of *P. latipinna* is amputated it fully regenerates in approximately two weeks. It follows the three phases of epimorphic regeneration *viz.* forming multi-stratified epithelial layer (wound epithelium), migration of mesenchymal cells near the amputation plane and their accumulation followed by proliferation of these mesenchymal cells to form the blastema and differentiation of the blastemal cells to replace its lost structures (Goss and Stagg, 1957; Santamaría and Becerra, 1991; Géraudie and Singer, 1992; Johnson and Weston, 1995; Poss *et al*., 2000a; Santos-Ruiz *et al*., 2002; Akimenko *et al*., 2003). Wound healing or wound epithelium formation is achieved within 12-24hours after amputation (hpa) and blastema stage is achieved in 24-48hpa. Finally, from 48hpa onwards, the blastema differentiates and

restores structures including blood vessels, bony rays, and connective tissue (Santamaria and Becerra, 1991; Johnson and Weston, 1995; Becerra *et al.,* 1996; Poss *et al.,* 2000b).

Several studies have been carried out in various teleosts (Morgan, 1902; Tassavva and Goss, 1966; Goss and Stagg, 1975; Santamaria and Beccerra, 1991; Geraudie *et al*., 1994). Also, many genes involved in fin regeneration are involved in fin development in fishes or limb development in other species as well (Laforest *et al*., 1998; Iovine, 2007). There is a long list of signalling molecules involved in zebrafish fin regenerative processes that contributes in the regulation of several cellular events. Thus, understanding the mechanism of regeneration in teleost caudal fin along with the lizard study will provide essential information which can be extended up to mammalian organ regeneration including humans (Masaki and Ide, 2007).

Few epimorphic regenerative studies in *P. latipinna* were carried out in our lab. Study includes the effect of FGF2 signalling on regenerating caudal fin with varied expression level of MMPs, which signifies their role during wound healing, proliferation and differentiation process (Saradamba, 2012). In another study, BMP signalling was also found to be crucial for regeneration (Rajaram, 2016).

The first aim of this study in the *P. latipinna* was to get protein expression profile during regeneration. For that two-dimensional gel electrophoresis was performed and it was found that approximately 200 spots were present in each regenerative stage. Comparison among them revealed nearly 100 peptides showing similar intensities and the remaining half were found to be altered. Out of these differentially expressing peptides across the stages of regeneration, two spots were selected for further studies based on their largest difference in expression between the stages studied. Upon identification of these two peptides, they were found to be, Keratin type I cytoskeletal 15 like and Myosin light chain-1. In order to look into their transcript level expressions, qPCR studies were conducted among the stages of regeneration in *P. latipinna* caudal fin. Significant change was noticed in expression of *krt15* at 24hpa i.e. the wound epithelium and *myl-1* in the differentiation stage (Chapter 3).

When detailed study of literature was carried out, it was found that several keratins are conserved evolutionarily. It has been documented that the chromosomal arrangement of Keratin8, Keratin18, and a second type II keratin, as a cluster of three genes, has remained conserved in vertebrate evolution (Krushna *et al*., 2006). Thus, its chances of being conserved up to mammals remained high. And this study can further be extended for deciphering the important functions of the Keratin type I cytoskeletal 15 like and Myosin light chain-1 during regeneration process. However, in the light of the current observation it could be construed that that Keratin type I cytoskeletal 15 like is playing an important role during wound healing and myosin light chain-1 is not involved in the wound healing and proliferative events of the regeneration but important during differentiation stage in teleost sailfin molly and northern house gecko.

Furthermore, epimorphic regeneration provides an excellent example of post-embryonic development and follows the same developmental dynamics as of an embryonic development to form a new structure. Their positional identity also plays important role towards formation of proximo-distal patterning and tissue differentiation in definite lineage (Bryant and Iten, 1976; Gardiner and Bryant 1989; Endo *et al.,* 2004). An important event of tissue remodelling is orchestrated by several proteases that digest the remaining tissues at the amputation site. Tissue remodelling is required to form the matrix for the cell-cell interaction, cell-tissue interactions and dedifferentiation process. Dedifferentiation involves the action of secreted proteinases for the formation of the undifferentiated cells. With the help of hydrolases and proteinases, extra cellular matrix (ECM) undergoes dedifferentiation and all the newly formed capillaries, fibroblast cells remain embedded in the loose ECM (Metcalfe and Ferguson, 2005). ECM is generally composed of fibrous structural proteins, specialised proteins and proteoglycans (Nagase and Woessner, 1999; Davis *et al*., 2000; Zhang *et al*., 2003). It provides the loose matrix containing fibronectin and fibrin to promote migration of wound keratinocytes (Schultz and Wysocki, 2009). Other than promotion of keratinocyte migration, ECM remodelling also occurs during major processes of development, morphogenesis, and wound healing, also found in case of disease state (Daley and Yamada, 2013). ECM remodelling has been reported in case of several animal models of regeneration

including zebrafish, *Xenopus*, newt and axolotl (Toole and Gross, 1971; Gulati *et al*., 1983; Tassava *et al*., 1996; Calve *et al*., 2010; Mercer *et al*., 2013, Govindan and Iovine, 2015). MMPs, the zinc dependent endopeptidases, play a significant role during the degradation and remodelling of ECM (Nagase and Woessner, 1999).

MMPs establish a family of more than 20 members (Birkedal-Hansen *et al*., 1993). Collagenases, gelatinases, stromelysins, matrilysins and membrane type MMPs (MT-MMPs), are types of MMPs and they are distinct with a wide range of substrate specificity. MMPs constitute three domains namely the ‘pre’ domain, the ‘pro’ domain, and the catalytic domain. A ‘pre’ domain gives signals for cellular export and a ‘pro’ or ‘propeptide’ domain is an inactive form of enzyme and by proteolytic activity on domain it activates enzyme (Vincenti *et al.*, 1996; Hulboy *et al.*, 1997). Enzyme is generally stabilized by two Zn^{2+} ions and two or three Ca^{2+} ions.

During epimorphic regeneration, several MMP family members were reported in various tissues and in different animals from fish to mammals (Grillo *et al*., 1968; Yang and Bryant, 1994; Miyazaki *et al*., 1996; Yang *et al*., 1999; Vinarsky *et al*., 2005; Delorme *et al*., 2012). Numerous events like angiogenesis, cellular migration, aggregation and apoptosis, were noted in which MMPs contribute to a major extent (Stamenkovic, 2003; Lemaitre and D'Armiento, 2006). Among all, MMP2 and MMP9 are gelatinases also referred as type IV collagenases. Their structure is unique because they contain three repeats of fibronectin-type II domain within the catalytic domain (Ganea *et al.,* 2007). It has been documented that gelatinases act on type IV collagen and on other components of ECM such as fibronectin, laminin, aggrecan, elastin, large tenasin-C and collagen I, V, VII and X (Nagase and Woessner, 1999; Davis *et al*., 2000; Zhang *et al*., 2003). Their significant role during regeneration was noted as their inhibition leads to scar formation (Vinarsky *et al*., 2005).

Due to their functional importance, we focus our study on their expression pattern during regeneration. To understand the regressive events and expression of MMP2 and MMP9 at a particular time, we conducted the study at protein and transcription level. In our lab, it was observed that proteolytic activity is always affected upon the inhibition of important key molecules during the process and eventually, delayed

growth or impaired restoration resulted (Pillai, 2012). Therefore, the normal expression profile of gelatinases needs to be identified. To achieve this goal, the respective stages of regeneration were collected and protein expression was checked through Western blot analysis followed by transcript analysis using quantitative real time PCR in regenerating tail of *H. flaviviridis*. Additionally, MMP2 and MMP9 were marked in the two-dimensional gel electrophoresis profile in accordance to their pI and molecular weight (Chapter 3). They were labeled in the gel picture and the change in the expression was noted upon the comparison with each other.

Results of the experiments in regenerating of *H. flaviviridis* using Western blot and qPCR depict the same result as we previously observed during the zymography study. Densitometric analysis of MMP2 showed elevated protein level during wound epithelium and blastema stage while a significant increase in the level of MMP2 was also found at transcript level at WE stage but was found to be down regulated at BL stage. For regenerated stage, protein levels were found high compared to resting stage but transcript levels were reported to be decreased. MMP9 study revealed up-regulation at both peptide and transcript levels at WE and BL stage but it was not found to be increased in REG stage at transcript levels but was almost comparable to resting stage at protein level (Chapter 4).

Parallel to this study the temporal expression pattern of MMP2 and MMP9 was also investigated in Sailfin molly, *P. latipinna* during caudal fin regeneration. From this study, the exact time point when activities of MMP2 and MMP9 peak could be determined. In order to achieve the goal, time points chosen were 0hpa, 1hpa, 2hpa, 3hpa, 4hpa, 5hpa, 6hpa, 12hpa, 18hpa, 24hpa, 60hpa and 5dpa. The selection of the time points was made based on our focus on the particular stage. Formation of the wound epithelium is an important step towards the successive regeneration process. Major tissue degradation and tissue remodeling occurs during the initial few hours. We had studied the expression level of MMP2 and MMP9 in detail with the 1hour time interval in the initial phase of study. Also, we were interested to know which gelatinases appear first during the process.

Hence, the gelatin zymography for gelatinases activity at each time point from the tissue extract was carried out. Heightened activity of MMP2 was observed only after 6hpa while MMP9 activity was marked its presence since 3hpa. During the time interval analysis, maximum activity of both the MMPs were found at 5dpa i.e. during early differentiation stage. Results also revealed the surges in the levels of MMP2 and MMP9 during the entire time course, suggesting the controlled expression of MMP2 and MMP9 during regeneration.

Significant presence of MMP9, in both animal models at WE and BL stages, consolidate the idea that these factors mediate the process of tissue remodelling. Several studies support our results that these are required throughout the process (Vinarsky *et al*., 2005). The controlled expression of MMP9, at particular time points i.e. at 18hpa and 24hpa, was found to be elevated again post WE stages. This can be explained by the fact that MMP9 is stimulated with the action of inflammatory molecules (Spiegel *et al*., 1996), secreted at the site of amputation to eliminate the infection or penetration of microflora via the exposed site of the tissue. It has been well documented that to inhibit such inflammatory mediators MMPs are required (Ito *et al*., 1996; McQuibban *et al*., 2001; Van Den Steen *et al*., 2003). There are several factors like tissue factor pathway inhibitor-2, a C-terminal fragment of the procollagen C-terminal proteinase enhancer protein (RECK), α2-macroglobulin and TIMPs (Barrett, 1981; Mott *et al.,* 2000; Herman *et al.*, 2001; Oh *et al.,* 2001) responsible for the regulation of MMPs leading to the controlled activity of MMP. Increased levels of MMP9 from 4hpa to 12hpa indicate its role in digestion of the basement membrane to provide a space for accumulation of proliferating and migrating cells. There are reports on contribution of MMPs in formation of wound epithelium by degrading basement membrane (Yang and Bryant, 1994; Miyazaki *et al*., 1996; Nagase and Woessner, 1999; Vinarsky *et al*., 2005).

In case of MMP2, the activity was found to be increased throughout the regeneration stages during *P. latipinna* caudal fin regeneration. Several notions of involvement of MMP2 during oncogenesis have been reported. MMP2 null mouse showed bone defects and significance of MMP2 in early stages of skeletal growth and development has been described too (Itoh *et al.*, 1997; Mosig *et al.,* 2007). BMP signalling is

another key pathway for formation of functional bone but its inhibition leads to impaired chondrogenesis. MMP2 was reported to be negatively regulating the inhibition of BMP signalling in the cancer cells (Laulan and St-Pierre, 2015). Collectively, it could be inferred that MMP2 is essential for inducing skeletal differentiation and hence, its activity was found high during early differentiation stage of tail fin in teleost fish. It could be construed that the MMP9 activity peaked a few hours before the wound epithelium formation so that digestion of the basement membrane of epithelial layer can be achieved for creating a route for the formation of wound epithelium. Thus, MMP9 and MMP2 are playing a vital role in regulating the process of regeneration in both the animal models studied, further supporting the evolutionarily conserved mechanism of regeneration.

To summarize, an attempt was made to find out the key molecules governing regeneration using advanced proteomic tools like two-dimensional gel electrophoresis followed by mass spectrometry and label-free detection method in two animal models, *Hemidactylus flaviviridis* and *Poecilia latipinna*. The analysis of the data obtained revealed similar expression pattern of peptides during various milestones of epimorphosis. Moreover, the temporal expression pattern of Keratin type I cytoskeletal 15, Myosin light chain 1/3 skeletal isoform, MMP2 and MMP9 also followed a strikingly similar trend during the course of appendage regeneration in both the animal models studied despite the vast difference in their evolutionary hierarchy. Overall, an apparent parallelism in the global protein expression pattern during appendage regeneration in the selected animal models was evident. It is therefore believed that the information presented herein will open up new avenues of research in regeneration.