CHAPTER 1

INTRODUCTION

In the later life, many invertebrate and vertebrate animals activate their development system to replace any of the body part or tissues which may have been lost. This post-embryonic development is called regeneration. It is now well accepted that every species exhibits some extent of regenerative capability. However, regeneration can be either complete or incomplete, depending on whether the lost tissue is completely replaced (resembling the original one) or it is just healed without being restored (Bryant and Fraser, 1988). The former phenomenon is a topic of intense research in the field of experimental and developmental biology since very long, due to the fact that humans lack the capability to restore body parts which have been lost due to injury or disease (Himeno *et al*., 1992).

Regeneration has been a subject of study since the eighteenth century. The first description of regenerative capacity was reported in *Hydra* by a Swiss zoologist Abraham Trembley in 1744 and from then on it attracted the attention of many biologists. *Hydra* is a freshwater cnidarian, and its regeneration takes place not only from tissue pieces excised from the body column but also from reaggregates of dissociated single cells (Noda, 1971; Gierer *et al*., 1972). They possess stem cells with high proliferative capacity, giving them an ability to reform their entire body from small fragments (Bosch, 2007). By the turn of the nineteenth century, the first comprehensive compilation of the works on regeneration was presented by T.H. Morgan in his classic treatise 'Regeneration' (1901). Planarians were found to exhibit an extraordinary ability to regenerate lost body parts. A planarian, which splits either horizontally or vertically, will regenerate into two separate individuals. In fact, even a fragment with few 10,000 cells can successfully regenerate into a new individual within one to two weeks (Montgomery and Coward, 1974). Beginning of 21st century, planarians gained the status of a model genetic organism to study the molecular mechanism of regeneration largely due to the works of Alejandro Sanchez-Alvarado

and Newmark (1998). The Large scale regenerative ability has also been reported in Echinoderms (such as the starfish), arthropods (such as the crayfish), many reptiles, and amphibians. Echinoderms are radially symmetric marine animals, which possess propensity of appendage regeneration wherein some can also regenerate internal organs and parts of their central nervous system (San *et al*., 2009). They undergo autotomy (Self-shedding of the appendage/body parts) of the appendage upon injury and regenerate it within three to four weeks time (Patruno *et al*., 2001).

In most fishes and salamanders, limited regeneration has been reported in tail or limb. Regeneration studies of the caudal fin in fish after experimental amputation has been appreciated for a long period of time (Morgan, 1901; Santamaria and Becerra, 1991). An outstanding ability of regeneration is exhibited by zebrafish to regenerate different parts of its body, including paired and unpaired fins, pectoral, pelvic, anal and dorsal fins, spinal cord and the heart ventricle (Poss *et al*., 2002; Poss *et al*., 2003; Kawakami *et al*., 2006; Nachtrab *et al*., 2011). Lazzaro Spallanzani had in 1768 reported that newts have the ability to regenerate their limbs. These are amphibians of the order urodele, having the highest regenerative ability among all tetrapods (Brockes *et al*., 2001). Histological and anatomical studies of amphibians were carried out later in the 19th century. Detailed experimental studies of urodele limb regeneration have been underway only since 1911. It was also discovered that salamanders could regenerate limbs and tail (including the spinal cord). Among the reptiles, Chelonians, crocodiles, and snakes are unable to regenerate lost parts, but many of the lizards, geckos, and iguanas possess regeneration capacity of a high degree. If a predator attacks their tail, they usually shed it off as an escaping mechanism and replace it within few days time. In addition to lizards, regeneration has been observed in the tails and maxillary bone of crocodiles (Brazaitis, 1981) but among the entire reptilian group, lizards possess the highest regenerative capacity (Bellairs and Bryant, 1985).

Other than cnidarians, planarians, fishes, amphibians, and reptiles, which are mentioned above and possess the most remarkable regeneration power; few other classes are also reported with regenerative ability, including members from arthropoda, annelida, aves, and mammalia. Arthropods are known to regenerate appendages following loss or autotomy, but it is restricted to moulting in some of the

animals; most crustaceans can regenerate throughout their lifetimes (Seifert *et al*., 2012). Their moulting cycles are hormonally regulated (Travis, 1955), but premature moulting can be induced by autotomy as well. Few other examples are; appendage regeneration, highly conserved in hemimetabolous insects, crustaceans (Das, 2015) and arachnids including scorpions (Nisani *et al.,* 2007).

Additionally, regeneration in annelids is well studied among various animals. *Chaetopterus variopedatus* and *Branchiomma nigromaculata* can are reported to restore their anterior and posterior body parts after latitudinal bisection (Hill, 1972). The regenerative relationship between somatic and germline stem cells has been studied at the molecular level in the polychaete, *Capitella teleta* (Giani *et al*., 2011). It is also proposed for Annelida that regeneration is either gained or lost during their evolution (Zoran, 2001) as few of the individuals can regenerate and few cannot. Some polychaetes like *Sabella pavonina* has been reported to undergo de-differentiation, transformation, and re-differentiation of cells to regenerate tissues (Zoran, 2001; Bely, 2014). Most annelids are capable of sealing their body via rapid muscular contraction upon amputation.

Moreover, birds are believed to have very limited regenerative abilities as adults and also during their embryonic development periods. Geese and ducks are capable of regenerating their beaks (Vorontsova and Liosner, 1960). Sidorova (1962) has reported liver regeneration in roosters. Birds can also regenerate the hair cells in their cochlea upon damage (Cotanche *et al*., 1994) and feathers (Hosker, 1936). Further, spontaneous regeneration has been induced in chick embryos by manipulating its cellular process using molecular biology techniques (Coleman, 2008). While in mammals, physiological regeneration including epithelial renewal (skin and intestinal tract), red blood cell replacement, antler regeneration and hair recycling has been observed (Kresie, 2001; Li *et al*., 2013). Few examples of mammalian regeneration are; Male deer lose their antlers annually and then through regeneration, they are restored (Price and Allen, 2004). Restoring the type of regeneration is rare among the mammals, but it is still reported in rabbits, pikas and African spiny mice (Seifert *et al*., 2012). A well-documented example of this type is the regeneration of the digit tip, distal to the nail bed in adult mice (Fernando *et al*., 2011).

Initially, it was thought that some tissues such as skin, re-grows quite readily in humans. Ongoing research in humans suggests this capacity in a variety of tissues and organs (Birbrair *et al*., 2013). Some of the examples of the physiological regeneration include formation of erythrocytes from haematopoietic stem cells in the bone marrow (Carlson, 2007) and regeneration of functional endometrium in females (Ferenczy *et al*., 1979). While in humans, one of the most studied regenerative responses is the hypertrophy of the liver following injury (Michalopoulo and DeFrances, 1997; Taub, 2004) wherein the function and mass of the liver is restored through the proliferation of existing mature hepatic cells, but the exact morphology of the liver is not regained. A cellular regeneration in humans has been observed in adult neurogenesis wherein hippocampal neuron renewal occurs (Spalding *et al*., 2013). In fact, cardiac myocyte renewal has also been found to occur in normal adult humans (Bergmann *et al*., 2009) following acute heart injury such as infarction (Beltrami *et al*., 2001). A well-observed reparative type of regeneration is fingertip regeneration (McKim, 1932; Muneoka *et al.,* 2008) and rib regeneration which is only partial and might take up to 1 year for completion (Satheesh *et al*., 2005).

TYPES OF REGENERATION

The first categorization of regeneration was described by Thomas Hunt Morgan in 1901 based on cell proliferation as ‘morphallaxis’ and ‘epimorphosis.' Morphollaxis involves the recreation of the lost body parts by the remodeling of existing cells; wherein there is little new growth. Such regeneration has been seen in hydras which utilizes stem cells found in the gastric region to regenerate itself or its lost structures (Bosch, 1998), while epimorphosis involves the dedifferentiation of adult structures to form an undifferentiated mass of cells that then becomes re-specified, an important characteristic of regenerating limbs of salamander (Figure 1).

Agata *et al*. (2007) used the terms distalisation and intercalation instead of mophollaxis and epimorphosis respectively, for the purpose of categorization based on positional information. Other types of regeneration described by Stoick-Cooper *et al*. (2007) include Compensatory growth**,** where uninjured parts of the organ compensate for the lost parts by growth (e.g., after removal of two lobes of the liver, the third lobe grows until the original mass of the liver is restored) and tissue regeneration where limited damage to an organ or tissue is restored by only one cell

type (e.g., skeletal muscle). Although restoration of lost parts of liver and muscle is less complicated than the complete restoration of the limb, it requires strict regulation of events for its success.

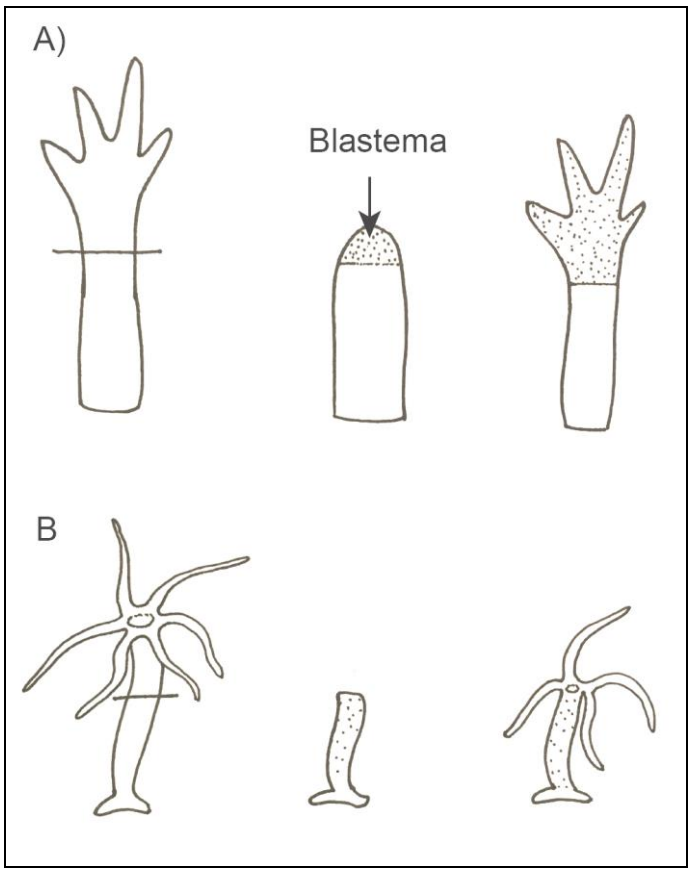

Figure 1: Classical examples of epimorphic and morphallactic regeneration. (A) Limb regeneration in amphibians; an example of epimorphic regeneration involves the formation of the blastema. (B) Hydra regeneration is categorized as morphallaxis (adapted from Agata *et al.,* 2007).

Morphallaxis

Morphallactic regeneration involves reorganization of the existing cells to form the lost structure; the remaining undifferentiated cells simply migrate to the site and differentiate into the specialized cells with little cell proliferation and growth that subsequently occurs to form regenerates which is smaller than the original ones (Cai *et al*., 2007). It is observed in species such as hydra. During this type of regeneration, a blastema is not formed at all (Figure. 1B).

Epimorphosis

The term epimorphosis is used to describe a regenerative process involving major changes in the differentiation states of cells participating in regeneration. Epimorphic regeneration is subdivided into two broad categories. In the first type, trans-differentiation e.g. Lens regeneration in urodele amphibians (Reyer, 1954) or limited dedifferentiation e.g. Liver (Michalopoulos and DeFrances, 1997) or proliferation and differentiation of stem cells e.g. Mammalian muscle is involved during regeneration (Uchida *et al*., 2000) to restore the lost or damaged part. While the second type of epimorphic regeneration involves the formation of blastema for the restoration. The blastema is similar to the early embryonic buds produced during vertebrate embryogenesis and forms a pool of mesenchymal progenitor cells required for proliferation and patterning of the regenerating part. This specialized structure formation has been described in planarians (Egger *et al*., 2007), molluscs (Flores *et al*., 1992), echinoderms (Thorndyke and Carnevali, 2001), crustaceans (Hopkins, 1993), teleost fish (Poss *et al*., 2003), urodele amphibians (Nye *et al*., 2003), larval anuran amphibians (Yokoyama, 2008), lizards (Clause and Capaldi, 2006) and in some mammals (Han *et al*., 2008).

It involves several exclusive biological events like 1) dedifferentiation of post-mitotic cells, 2) activation of multipotent progenitor cells, 3) cell proliferation, 4) pattern formation and 5) trans-differentiation of specialized cells (in some cases) to regain the lost body part (Poss *et al*., 2002; Tanaka, 2003; Brockes and Kumar 2005; Alvorado and Tsonis, 2006). Epimorphic regeneration is achieved by following four sequential steps: (1) formation of a wound epidermis, in which the amputation site is covered by epithelial cells, (2) disorganization and dedifferentiation of mesenchymal tissue near the wound, (3) formation of undifferentiated cell mass, known as the blastema, and (4) proliferation of the dedifferentiated cells to reform the lost organ (Figure. 2).

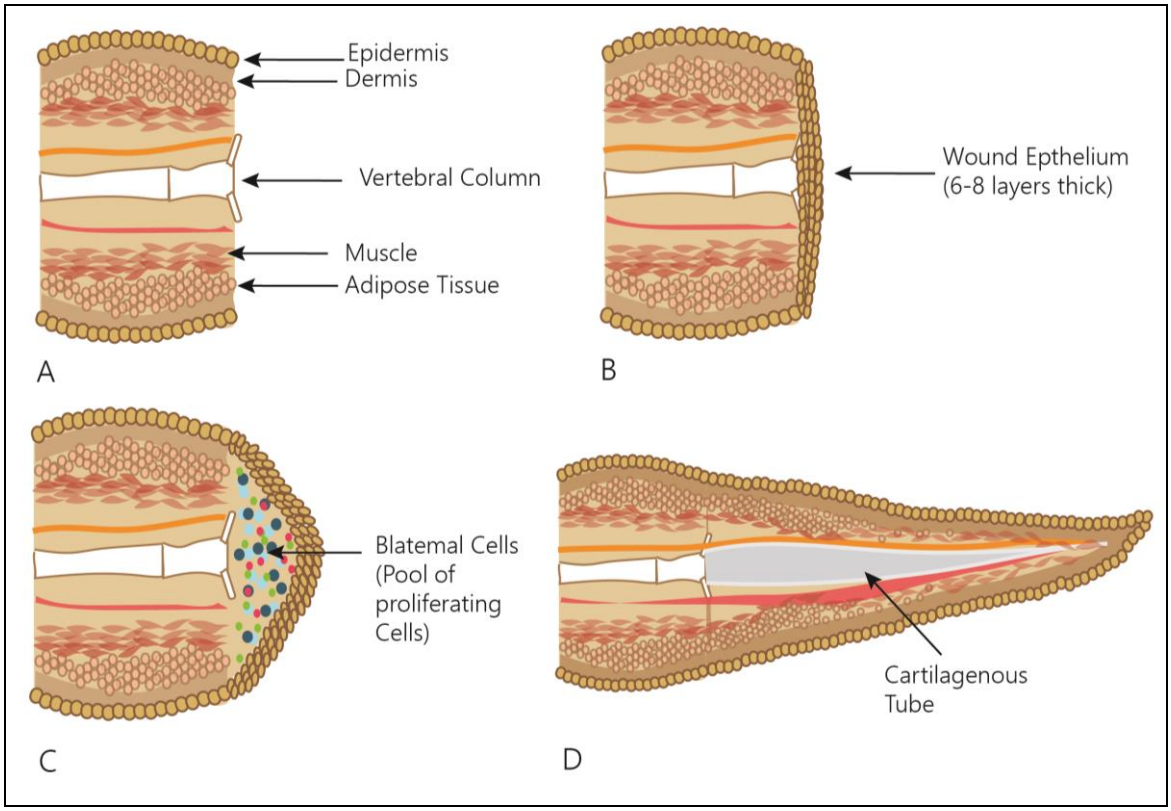

Figure 2: Schematic representation of epimorphosis in Lizards Tail: Formation of the wound epithelium is one of the first steps in regeneration and precedes blastema formation and cell proliferation. A) represents the amputated tail followed by B) wound epithelium formation which leads to C) Blastema formation and D) represents regenerated tail.

Further, Susan V. Bryant and David M. Gardiner divided limb regeneration into three phases: wound healing, de-differentiation, and redevelopment (Bryant *et al*., 2002; Gardiner *et al*., 2002); wherein the redevelopment phase completely mimics embryonic limb development. Epimorphic regeneration proceeds in a well-defined sequence of stages as explained in the following description.

STAGES OF EPIMORPHIC REGENERATION

Epimorphic regeneration is therefore summarised as an outcome of three well-defined stages:

1) Wound epithelium stage: Multilayered epithelium covers the wound
2) Blastema stage: Pleuripotent cells accumulate underneath the epithelial layer
3) Growth and Differentiation stage: Blastemal cells begin to differentiate as per their cell fate, forming a new organ.

A more detailed description of the said stages is taken up below.

Wound Epithelium Stage

Shortly after amputation, due to the migration of epithelial cells around the amputation site, the exposed mesenchyme is covered, forming a wound epithelium (WE) (Call and Tsonis, 2005). The time taken for complete wound closure varies among animals and depends on the size of the animal and hence on the size of its severed organ. It completes rapidly in smaller animals (Carlson *et al*., 1998). Factors responsible for the migration of the epithelial cells are still unknown, but the development of new skin is eventually required for the regeneration. Skin regenerates without scarring and releases signals for the expression of early stage genes. Few genes like *MMP-9* and *msx-2* are independently expressed prior to wound closure and do not depend on wound epithelium for their induction (Bryant et al., 2002). In fact, the *MMPs* (matrix metalloproteinases), which are responsible for the matrix digestion, contribute to the formation of the WE (Call and Tsonis, 2005; Vinarsky *et al*., 2005). It must be mentioned that continuous expression of such genes is inhibited by the grafts of mature epithelial cells (Carlson *et al*., 1998; Gardiner *et al*., 1999; Yang *et al*., 1999), indicating that adult skin may be detrimental to regenerative repair. The wound epithelium, after completely covering the amputation site, thickens by proliferation to a multilayered structure called the Apical Epithelial Cap (AEC). The cap shares functional similarities with the Apical Ectodermal Ridge (AER) of developing limb bud of amniotes (Summerbell, 1974; Saunder *et al*., 1976; Saunders, 1998). Such structural and functional maturation of the WE into the AEC is brought about by various factors present locally such as the Wnt/β-catenin pathway members (Poss *et al*., 2000a; Kawakami *et al*., 2006; Buch *et al.,* 2017).

Importantly, AEC maturation is accompanied by major changes in the differentiation states of cells immediately adjacent to the plane of amputation. Such changes mark the beginning of blastema formation, and they lead to the formation of a pool of cells under the AEC, from which all of the regenerating tissue will derive.

Blastema Stage

A precise definition of blastema (BL) may not be available, but morphologically it can be recognized as a stout cone shaped outgrowth, formed at the site of amputation.

The presence of undifferentiated cells has been clearly observed in histological sections of the blastema (Agata *et al*., 2007; Alibardi, 2009).

For investigating the origin of the blastemal cells, several studies have been conducted. The planarians possess pre-existing stem cells called neoblasts contributing to the formation of blastema (Baguna and Slack, 1981) while in axolotls, blastema arises by reprogramming and dedifferentiation of pre-differentiated cells (Namenwirth, 1974; Kintner and Brockes, 1984; Casimir *et al*., 1988; Lo *et al*., 1993; Echeverri *et al*., 2001; Brockes and Kumar, 2002; Echeverri and Tanaka, 2002). However, activation of resident muscle stem cells and transdifferentiation has also been reported (Morrison *et al*., 2006; Kragl *et al*., 2009). Thus, dedifferentiation, trans-differentiation and stem cell activation, all contribute to the formation of the blastema.

Signals from the WE are known to induce the formation of the regenerative blastema. Thus, shortly after wound epithelium stage regenerating blastema is formed. Several factors from the newly formed epidermis induce blastema formation. Very first evidence of the formation of blastema from the skin was reported with the expression of HoxA9 and HoxA13 from the distal stump of an amputated axolotl limb (Gardiner *et al.*, 1995). Blastema stage depends on signals from wound healing stage, but mature skin grafting inhibits the transition of wound healing stage to blastema stage. It is not fully known which signals are required for this transition. Nevertheless, it has been found that blastema formation depends on the nerves present and the signals derived from them. Blastema progression and proliferation also depends on the enervation. Denervation of a limb or removal of the axons inhibits the formation and progression of the blastema (Tsonis, 1996). Thus, nerve dependency is an essentiality for blastema formation. Several neurotrophic factors generated from the neurons stimulate cell proliferation (Mescher and Tassava, 1975; Maden, 1978; Mescher, 1996; Nye *et al*., 2003). Several factors influencing blastemal cell proliferation, like the FGFs and Dlx3, are downregulated due to denervation (Mullen *et al*., 1996; Cannata *et al*., 2001; Christensen *et al*., 2001). Many other neural factors like iron binding protein transferrin (Mescher and Munaim, 1984; Munaim and Mescher, 1986), GGF (neuregulin) (Wang *et al*., 2000) and FGF-2 (Mullen *et al*., 1996) have been identified. Few factors are independently expressed in the blastema

regardless of the denervation. Msx-1 is one such factor (Park *et al*., 2009). As already stated above, it is the blastemal pool of cells that grows through rapid proliferation and affects the actual morphogenesis of the appendage.

Growth and Differentiation Stage

There is ample of evidence supporting the idea of undifferentiated blastema, behaving as a developing limb bud of an embryo as the pattern of cellular contribution to development is almost identical for regenerating and developing limb (Muneoka and Bryant, 1984). Gardiner and Bryant (1998) have demonstrated that the spatial and temporal patterns of gene expression during blastema stages of regeneration are comparable to those during limb development of urodeles as well as other vertebrate embryos. Moreover, newly generated cells inherit a particular positional identity from the cells or from the surrounding environment and drive towards a particular pattern needed to form the complete structure (Brockes, 1997). Thus, complete morphogenesis is achieved during this stage.

In this phase, retinoic acid has been identified from several experiments and plays an important role in the proximodistal and dorsoventral patterning during axolotl limb regeneration (Ludolph *et al*., 1990). It acts on cell surface molecule Prod 1 (proximodistal-1), a newt CD59 homolog, and is an important factor determining the proximodistal identity of cells of the developing limb (da Silva *et al*., 2002).

The effect of artificial denervation during redevelopment stage is not as significant as that in the first two stages, but denervation of the late blastema ceases the outgrowth of the regenerating appendage. This may be because nerves provide signals that are required for blastema growth and its continuous dedifferentiation and denervation could block the interaction between HoxA9/13 in blastema, resulting in truncated stump of regenerating appendage (Stocum and Cameron, 2011). From this point of view, it has been suggested that once the process progresses to morphogenesis or redevelopment, it is very similar to that in embryogenesis.

FEW QUESTIONS OF EPIMORPHIC REGENERATION

The beauty and extraordinary complexity of regeneration has amazed scientists and

attracted them to explore this complex process. As a result, the field of experimental, developmental biology started. The principle questions of epimorphic regeneration asked by scientists from the past hundred years have been about (1) the origin of the blastema, (2) the mechanism of histolysis that liberates regenerative cells from their tissue organization, (3) the mechanism of dedifferentiation and accumulation of these cells to form a blastema, (4) the mechanism of blastema growth, (5) the models and mechanism of blastema patterning, (6) factors which are 'regeneration specific' involved in initiating this process at molecular level and (7) loss of regenerative power in adult birds and mammals (Stocum and Cameron, 2011).

Insight into the regeneration processes would yield clues about this specific response. A study highlighting the dissimilarities between regenerating non-regenerating organisms will be most helpful in this regard. Despite all the interest of researchers, little is known about the regenerative events in cellular, molecular and mechanistic terms.

Few of the research highlights in the field of epimorphic regeneration are as follows:

In the case of tetrapod limbs or fish fins, the signalling pathways that initiate and control appendage development are reactivated to promote the regenerative process, which is a post embryonic process. Several key signalling pathways identified and studied in the various regeneration models are FGF, Wnt/β-catenin, RA, Shh, activin and BMP signalling (Denis *et al.,* 2016; Shibata *et al.,* 2016; Nguyenm *et al.,* 2017).

A factor LEF-1 (lymphoid enhancer-binding factor 1), a Wnt signalling pathway member required during mammalian embryonic development, was also observed in wound epithelium stage in zebrafish fin regeneration (Poss *et al*., 2000a). During limb regeneration, Dkk1 (an inhibitor of wnt signalling pathway) blocks fin regeneration by inhibiting Wnt and FGF signalling both (Kawakami *et al*., 2006). Taken together, the reports indicate the importance of canonical *wnt* signalling in epimorphic regeneration. Wnt signalling is also required for blastema formation and regenerative outgrowth, supported by an elegant series of experiments (Stoick-Cooper *et al.,* 2007; Tal *et al*., 2010). Gospodarowicz (1976) reported the role of FGF as a

mitogenic factor and its significance in wound healing, blastema formation, and regenerative outgrowth. During blastema stage, *fgfr1* activates *shh* expression and its inhibition down-regulates *msx* and *shh* expression (Poss *et al.,* 2000b; Lee *et al.,* 2005; Thummel *et al.,* 2006; Lee *et al.,* 2009) while *fgf24 (wfgf)* is expressed during wound epithelium stage (Poss *et al.,* 2000b). During patterning in zebrafish fin regeneration, RA signalling (White *et al.,* 1994; Geraudie *et al.*, 1995; Geraudie and Ferretti, 1997) and Activin-βA are also involved (Jaźwińska *et al*., 2007). Shh (sonic hedgehog) is important to provide anterior-posterior axis information for the regenerating limb (Riddle *et al*., 1993; Roy *et al*., 2000) and controlled expression of shh, Patched 1, and bmp2 are responsible for skeletal regeneration and patterning in zebrafish (Laforest *et al*., 1998) and their signalling inhibition alters regenerative process (Quint *et al.,* 2002; Smith *et al.,* 2006)**.**

Nowadays, sophisticated genetic and molecular tools are available for analysis, and with these techniques, identification of various proteins and transcription factors involved in the various stages of regeneration was initiated.

Stem cells research has played an important role in regeneration biology. Mammalian adult fibroblasts have been induced to assume pluripotency, and these cells, called iPSCs (induced pluripotent stem cells) have similar property as those of embryonic stem cells (ESCs). They were produced by transfection of fibroblasts with four transcription factor genes *OCT4, SOX2, c-Myc, Klf4* (Takahashi *et al*., 2007) or *Oct4, SOX2, NANOG, and Lin28* (Yu *et al*., 2007). Among these, three transcription factors, *klf4, Sox2, c-Myc*, are upregulated during blastema formation in regenerating newt limbs and lens (Maki *et al*., 2009). Upregulated Lin28 protein was also detected during blastema formation in regenerating axolotl limbs (Rao *et al*., 2009).

Additionally, 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-sequence1), *Shh* enhancer, has been observed in *Xenopus* and moderately in Axolotl and Newts (Yakushiji *et al*., 2007).

Attempts to find out important regulators of regeneration have been made in various models including zebrafish, axolotl, and urodeles. To date, most research on naturally evolved epimorphic regeneration has focused on anamniotes including zebrafish and newts. Although explored in the context of ecological costs and benefits, less is known about the sequence of cellular and tissue level events of lizard tail regeneration or other reptilian members.

Many reptiles possess the ability to replace a lost tail through epimorphosis. Regeneration of the tail and jaws of crocodilians and the shell of turtles (Bellairs and Bryant, 1985; Carlson, 2007) are also known. Reptiles can be considered as a model with intermediate regenerative ability – lower than that of cyclostomes, fish, and amphibians but higher than amniotes. When we see the regenerative ability in lizards, they can restore nerve cells, part of a lower mandibular axon, parts of the limb and tail (Simpson, 1961; Bryant, 1970; Bellairs and Bryant, 1985). Although, the ability for caudal regeneration varies in different species of lizards they can repair large amputations of the mandibular and maxillary arch with the initial production of a cartilaginous tissue which later calcifies. Eye lens can partially regenerate, and a good repairing efficiency is present in the optic nerve for re-establishing anatomical connections with specific region of optic tectum (Beazley *et al*., 1997; Dunlop *et al*. 2004). Bone fractures are efficiently repaired by two different mechanisms; first with the formation of cartilage in the long bone (Alibardi, 2010) and secondly repair of dermal bones by the formation of osteoblasts, without involving secondary cartilage production (Irwin and Ferguson, 1986). Regeneration is not observed in snakes except that during for moulting of skin (Maderson 1971; Maderson *et al*. 1978; Smith and Barker 1988). 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). Even so, reptiles as models of regeneration have been completely underestimated and neglected for unclear reasons.

LIZARD AS A MODEL FOR REGENERATION STUDIES

Lizards (Geckos) are the closest group of organisms, in terms of evolutionary hierarchy to mammals that have an ability to replace a lost body part as large as the

tail. Although reptiles and mammals differ from each other in many aspects, the similarity between their histological features is definitely more than that between mammals and amphibians (Alibardi, 2009), making reptiles an attractive model to study tissue and organ regeneration.

Lizards represent the largest group of reptiles together with the snakes and amphisbaenas (Evans, 2003). Around 3300 species of tailed, largely medium sized diapsid derived species forms a group. They evolved from the mid-Triassic era, but fossil records are available only from the early Jurassic age. The presence of numerous vertebrae has always been a characteristic feature of early history (Bellairs and bryant, 1985; Evans, 2003). Lizards possess fracture planes through the vertebrae along which they can release their tail, under certain stimuli like grabbing, biting or attack by predators as depicted in figure 3. This is considered to be associated with the evolution of regeneration as a causal phenomenon (Vitt 1983; Reichman 1984; Goss 1987; Maginnis 2006). Generally, regeneration occurs from the intervertebral plane of autotomy but in some cases, it occurs from the intra-vertebral plane as well (Arnold, 1984).

Figure 3: Schematic representation of Lizards Tail. Black arrow head represents epidermis below which is the dermis shown by orange arrow head followed by adipose tissue by the blue arrow head. Below the adipose tissue is the muscle depicted by the red arrow head. Green, Purple, and yellow arrow heads show the neural spine, spinal cord, and vertebral column respectively. Brown and pink arrow heads are the caudal artery and caudal vein respectively.

In any vertebrate system, large wounds are often fatal and are not subjected to the selective pressure for regeneration. Rather small wounds are subjected to a high selective pressure to regenerate or repair so as to avoid the penetration of microbes by exerting prolonged inflammatory response, resulting in scar formation (Maginnis, 2006). As lizard tail is important but not an essential organ for survival, wounds of the

intermediate size can be repaired. Also, lizard species lose their social rank due to the lack of the tail (Fox and Rostker, 1982) Further, tail is a storage organ for fats (upto 50% of the body fat) and thus by rapid tail regeneration, chances of survival are improved (Fitch, 2003; Clause and Capaldi, 2006). This remarkable process of tail regeneration (a non-vital organ) highlights the biological or morphological replacement to favor metabolic survival, the speed of movement, prehensility, social behavior, etc. (Alibardi, 2010).

The ability of lizards to regenerate lost parts, albeit limited, is more like their vertebrate ancestors namely fish and amphibians. However, lizards replace their lost appendage (tail) with an un-segmented tail, quite contrary to the metamerically segmented original tail. Nonetheless, this new tail is good enough for the animal to regain its social acceptability and for its primary functions such as fat storage.

In the group of lizards, leopard gecko *Eublepharis macularius* was used as amniote model to investigate the anatomical and histological events that characterize tail regeneration (McLean and Vickaryous, 2011). Recently, the first genomic database of the reptilian group was achieved using Green anoles, *Anolis carolinensis* (Hutchins *et al.,* 2014). The northern house gecko, *Hemidactylus flaviviridis* has been used as a regeneration model in our department since many years (Kumar and Pilo, 1994; Pilo and Suresh, 1994; Pilo and Kumar, 1995; Sharma and Suresh, 2008; Suresh *et al.,* 2009; Buch *et al.,* 2017).

Hemidactylus flaviviridis (Rüppell, 1835), from Gekkonidae family which has almost 90 species, is also named as yellow-bellied house gecko, due to its yellow ventral skin and readiness to adapt to and coexist with humans. They are oviparous; can grow up to 18-20cms in length, the body is covered with small keeled scales, showing distinct variation in the body color. At night, it is typically greyish, olive-brown in color with indistinct bands on the back while at day time it usually much darker in color with chevron shaped bands. Body and the head are usually flat, and tail has enlarged tubercles (wart like bumps and ridges) along the dorsal side (Nanhoe and Ouboter, 1987; Halliday and Adler, 2002; Gardner, 2005; Bartlett and Bartlett, 2006). Their toes possess broad pads which are covered with small scales called scansors, each scansor has up to 150,000 microscopic, highly branched, hair-like

structures, known as setae, and at the toe tip, small claws are present (Halliday and Adler, 2002; Gardner, 2004; 2005).

Hemidactylus flaviviridis has been used in our department to study the mechanisms of regeneration since over five decades. Various aspects of regeneration such as histological, biochemical and metabolic alterations in this gekkonid lizard have been addressed (Kumar and Pilo, 1994; Pilo and Suresh, 1994; Pilo and Kumar, 1995; Yadav *et al*., 2012). Studies have revealed that some growth factors and neural peptides 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). However, there could be many more regulatory proteins working in unison to facilitate various cellular events during regeneration, and these proteins need to be identified. Therefore, a detailed study on the overall expression profile during tail regeneration was deemed necessary. A parallel study in this direction was also carried out using teleost fish, *Poecilia latipinna* to create a picture of the evolutionary trend in appendage regeneration through epimorphosis.

FISH AS A MODEL

As with lizards, fish also present an excellent model to study epimorphic regeneration. More than 200 years ago, Broussonet (1786) reported that a fish could completely regenerate its fins after amputation. Following such observations made on numerous teleost fish, most of the research that followed in this area was carried out in zebrafish due to its accessibility, fast and robust regeneration, its simple architecture and above all, its widespread use as a model for developmental biology research.

The caudal fin of teleosts is composed of several segmented bony rays named lepidotrichia (Montes *et al*., 1982; Becerra *et al*., 1983; Géraudie and Singer, 1992) and inter-ray mesenchymal tissue enclosed by an epidermis. As shown in figure 4 each lepidotrichium consists of two concave hemirays which generate an inner space filled with the mesenchymal cells. There is a cluster of small, fusiform, rigid and slender spicules called actinotrichia at the margin of the lepidotrichia towards the edge of the tail fin, which supports the border of the tail fin (Becerra *et al*., 1983). Blood vessels, nerve axons are found in both intra-ray and inter-ray tissues (Poss *et*

al., 2003). Once the caudal fin is amputated, it fully regenerates in approximately two weeks (this may vary with the specific fish). It follows the three phases of epimorphic regeneration similar to those described above for the tetrapods. It starts with the formation of a multistratified epidermal layer (wound epithelium) by migration of mesenchymal cells near the amputation plane and their accumulation, and is 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; Wiley *et al.,* 2015).

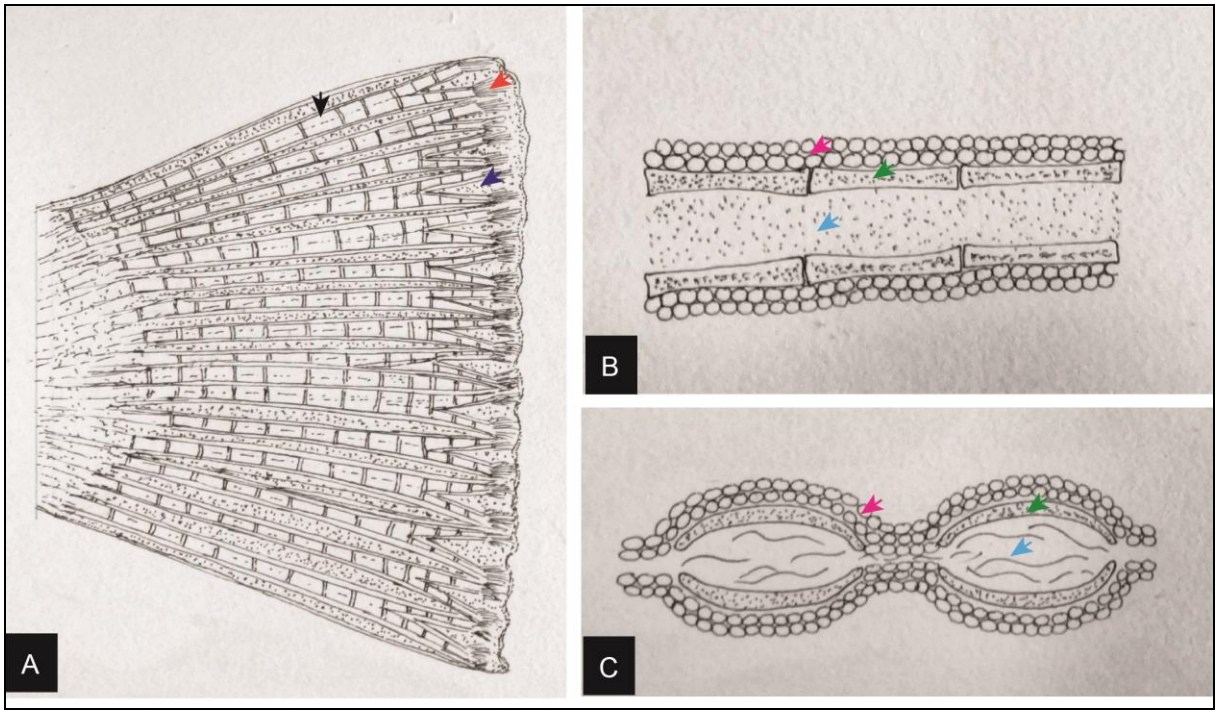

Figure 4: Schematic representation of caudal fin of fish: A) Entire fin showing lepidotrichia marked by a black arrow head, actinotrichia by orange arrow head and purple arrow head shows the inter-ray tissue. B) Longitudinal section of lepidotrichium followed by C) Transverse section of lepidotrichia. Pink arrow head represents epidermis; green arrow head shows the hemiray and light blue arrow head represents mesenchyme.

Remarkably, teleost fish can regenerate almost all the body parts. Significant regenerative proficiency has been reported in a variety of tissues and organs including scales, muscles, spinal cord and heart along with their fins (Akimenko *et al*.,

2003; Poss *et al*., 2003). Fish fins can also be considered as homologous to tail and limb of vertebrates, and their mechanisms can be shared among them (Hinchliffe, 2002). Thus, understanding the mechanism of regeneration in teleost caudal fin along with the lizard will provide essential information that can be extended up to organ regeneration in mammals including human. Moreover, many genes involved in fin regeneration are also important in fin development and limb development in other species (Laforest *et al*., 1998; Iovine, 2007; Masaki and Ide, 2007).

Studies on fin regeneration have been carried out in various teleost fishes like Gold fish (Morgan, 1902; Santamaria *et al*., 1992; 1996; Mari-Beffa *et al*., 1999), Minnows and Blennies (Morgan, 1906; Goss and Stagg, 1975), *Opalina gouramis* (Tassavva and Goss, 1966), Sword tail fish (Zauner *et al*., 2003) Tilapia (Santamaria and Beccerra, 1991), Trout (Alonso *et al*., 2000) and Zebrafish (Geraudie *et al*., 1994; Johnson and Weston, 1995; Mari-Beffa *et al*., 1999; Poss *et al*., 2003). However, considering the easy and ready availability, the teleost fish Sailfin Molly, *Poecilia latipinna* (Lesueur, 1821), was used in the present study. These can be easily maintained in large numbers, and their fins are structurally simple and provide ease of experimental procedures like an amputation.

The sailfin molly, *Poecilia latipinna,* formerly described and named *Mollienesia latipinna* by Charles Alexandre Lesueur in 1821, is a fusiform shaped small fish (15-53 mm total length) with a small head and upturned mouth (Robins, 2003). The molly is also popular in the aquarium trade and is available in a wide variety of colours through domestication. Of these, the albino molly was used in the present studies. As far as the events of regeneration are concerned, they go on the same lines as the generalised scheme described above. Under our conditions of the study, wound healing or wound epithelium formation is achieved by 24 hours post amputation (hpa); blastema stage is achieved by 48 hpa, and this is followed by a smooth transition into the growth and development phase which extends from 48 hpa to the completion of regeneration.During this phase, structures including blood vessels, bony rays, and connective tissue are restored (Santamaria and Becerra, 1991; Johnson and Weston, 1995; Becerra *et al.,* 1996; Poss *et al.,* 2000b).

AIM OF THE STUDY

Epimorphosis involves several events like programmed cell death, extracellular matrix remodelling, cell migration leading to the formation of a functional wound epidermis, accumulation and proliferation of undifferentiated cells beneath the dermal layer and redifferentiation of cells to restore the lost structure. To date, most the understanding of epimorphic regeneration is based on research conducted on anamniote models of regeneration like zebrafish, newt, salamander and anural tadpoles. Very little is known about the sequence of cellular and tissue level events of lizard tail regeneration and also about the involvement of various regulatory factors acting at specific stages of amniote appendage regeneration. **The present study was, therefore, undertaken to evaluate the protein expression pattern during the major milestones of epimorphic regeneration of the tail in *Hemidactylus flaviviridis.*** With an aim to throw light on the evolutionary pattern of the process among the amniotes and the anamniotes, the same experiments were performed in the regenerating caudal fin of teleost fish *Poecilia latipinna*. This aim was fulfilled by the following specific objectives.

1. **Evaluation of the global expression profile of peptides** during tail or fin regeneration using two-dimensional gel electrophoresis. Regenerating tail or fin tissue from the key stages of regeneration *viz*. wound epithelium, blastema, and differentiation (fully regenerated tail) were used. An overall number of peptides expressed was analyzed at each stage and comparison was made among all samples. Further, peptides expressed distinctly were taken into consideration for their identification. Peptides differentially expressed during the wound epithelium and blastema stages were selected for the **identification through sequencing**. Similarly, peptides present in the intact (resting) tail and fully redeveloped structures but not in the proliferative stages i.e. WE and BL, were selected for sequencing as well. Sequence analysis and identification were carried out using the nano LC-MS/MS technique. To understand the regulation of these peptides, their expression was assessed at transcription level using real time PCR. This objective was parallelly pursued in *P. latipinna* as well.

2. Matrix digestion and remodeling is one of the very significant processes for the formation of the wound epithelium. MMPs are already reported to play a pivotal role in ECM remodelling during regeneration in various vertebrate models, and it is well perceived that their inhibition leads to impaired growth. In the current study, the temporal expression pattern of MMPs was attempted using *H. flaviviridis* as well as *P. latipinna*. Herein, **the temporal expression patterns of MMP2 and MMP9** were worked out at both transcriptional and translational levels using western blot analysis and real time PCR.