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Introduction

Regeneration is the process of restoring the lost tissue or structure by reactivation of the developmental process in post-embryonic life (Gilbert and Barresi, 2016). The potential to regain the lost body part has intrigued many researchers who tried to explore and understand the mechanism of regeneration. Due to the complexity of the process, even after several decades of work by scientists in the field of regeneration, limited progress has been accomplished towards unraveling the phenomenon. However, understanding the process of regeneration is of utmost importance as it provides an impetus to the field of regenerative medicine. Many organisms possess the ability to regenerate their entire body, or body parts, however we humans lack the ability to regenerate major structures and hence, for our own selfish motives, the field of regenerative medicine has beguiled a lot of attraction. Over the course of evolution, humans might have lost their regenerative capability, however understanding how regeneration works and whether it can be evoked remains unanswered till date.

HISTORY OF REGENERATION STUDIES

Regeneration is not a recent field, instead, it is one of the oldest fields in experimental biology (Vorontsova and Liosner, 1960; Dinsmore, 1991). Aristotle and Pliny were the first to describe in their writings about regeneration during ancient times, however, the scientific observations of regeneration were made and reported by René-Antoine Ferchault de Réaumur in 1712. Réaumur studied and gave a comprehensive description of limb regeneration in crayfish. Réaumur drafted the controversial theory of preformation which hypothesized that the limb regeneration occurred from the expansion of the tiny preformed limbs that resided at the base of the limb which was disproved later. Nonetheless, the next half of the century saw a surge of seminal investigations on regeneration in a variety of organisms. Work on hydra was done by Abraham Trembley in 1744 where he cut the hydra in two and saw them regenerating which was a groundbreaking discovery at the time. Charles Bonnet in 1745 studied regeneration in annelids, and Spallanzani (1769) on amphibians and finally Pallas (1776) investigated planarian regeneration which even today is being studied extensively. The phenomenon of regeneration was very popular amongst the philosophic discussions at that time. In the late eighteenth century, members of French nobility took scissors and amputated the snail heads in

their garden just to observe them regenerating their head. Such was the impact of regeneration during that time.

However, by the early nineteenth century, naturalist explained regeneration in many animals but their observations were merely confined to morphological changes. It was only after Matthias Schleiden and Theodor Schwann (1838-39) gave the cell theory and with the development of histology techniques, obtaining a clear picture of regeneration became achievable. Planarian regeneration studies were conducted by Charles Darwin on one of his famous voyages. Weismann (1892) expanded the then available knowledge on morphogenesis on limb regeneration in the pre-Mendelian era of genetics. It was Thomas Hunt Morgan (1901), at the beginning of the nineteenth century, who rekindled interest in the field regeneration biology before he left the field to pursue his famous studies on the genetics of Drosophila. In the twentieth century, a lot of studies on a variety of species were conducted, however interest in mammalian regeneration faded because of the apparent lack of ability to regenerate any major tissue or organ. Meanwhile, Child (1941) gave the theory of metabolic gradient and work of many scientists in the field of invertebrate regeneration was based on this. It is notable that the work on amphibian limb regeneration moved from just the description to actual experimental studies wherein specific components in regeneration and the role of dedifferentiation along with morphogenesis was besieged.

During the World War II period, a plethora of studies were directed towards stimulating limb regeneration in frogs. However, due to lack of knowledge and technological advancements in the latter half of the nineteenth century, the interest started to fade. It was only towards the end of the century when substantial evidence for morphogenesis came into light and thus once again evoked attraction towards this field. The field of regeneration gained even more attention during the molecular revolution but surprisingly the knowledge on molecular events is still not clear. Even after so many years of work on regeneration, one still needs to unravel the exact mechanism. With the advent of newer technologies and sophisticated instruments the expectations are high. Not only this, stem cell biology is now promoting the field of regeneration and a substantial breakthrough is expected.

Nonetheless, to exemplify the mechanism of regeneration one must know how many different types of regeneration are known and accepted worldwide. Regeneration is not a single process, rather a conglomerate of many developmental processes getting activated in a nested manner. Hence, there are different types of regeneration based on the complexity and the route through

which the lost part is regained. The subsequent section defines the currently recognized types of regeneration.

TYPES OF REGENERATION

For years, the literature has shown extensive discussion on defining the types of regeneration. Such classifications have their value when well-understood processes are delineated and compared. But unfortunately, the understanding of the regenerative process is so poor it has become very diffeicult to assign any particular category.

Based on the path taken by a regenerating system, regeneration has been broadly classified into two major types, viz., Morphallaxis and Epimorphosis. This classification has been in place since nineteenth century and is mainly based on morphological evidences. However, now instead of the two types of regeneration that were earlier accepted, in total four types of regeneration have been established based on the cellular alteration that takes place at the site of injury which are explicated below.

1. Stem-cell mediated regeneration.

   An organism would possess certain resident stem cells which allow the organism to regrow definite tissues or organs that have been injured or even lost (Vining and Mooney, 2017). Regrowth of hair shafts from the follicular stem cells is one such example, along with continuous replacement of blood cell by haematopoesis in bone marrow (Figure 1.1 and 1.2).

2. Epimorphosis.

   In some species, adult structures can undergo dedifferentiation to form a relatively undifferentiated mass of cells (a blastema) which then redifferentiates to form a structural and functional duplicate of the original body part or tissue, which has suffered a physical insult (Suzukui et al., 2006). Such regeneration is characteristic of regenerating amphibian limbs (Figure 1.1 and 1.2).

3. Morphallaxis.

   Here, regeneration occurs through the repatterning of existing tissues (transdifferentiation), and there is little new growth (Agata et al., 2007). Such regeneration is seen in the hydra (Figure 1.1 and 1.2).

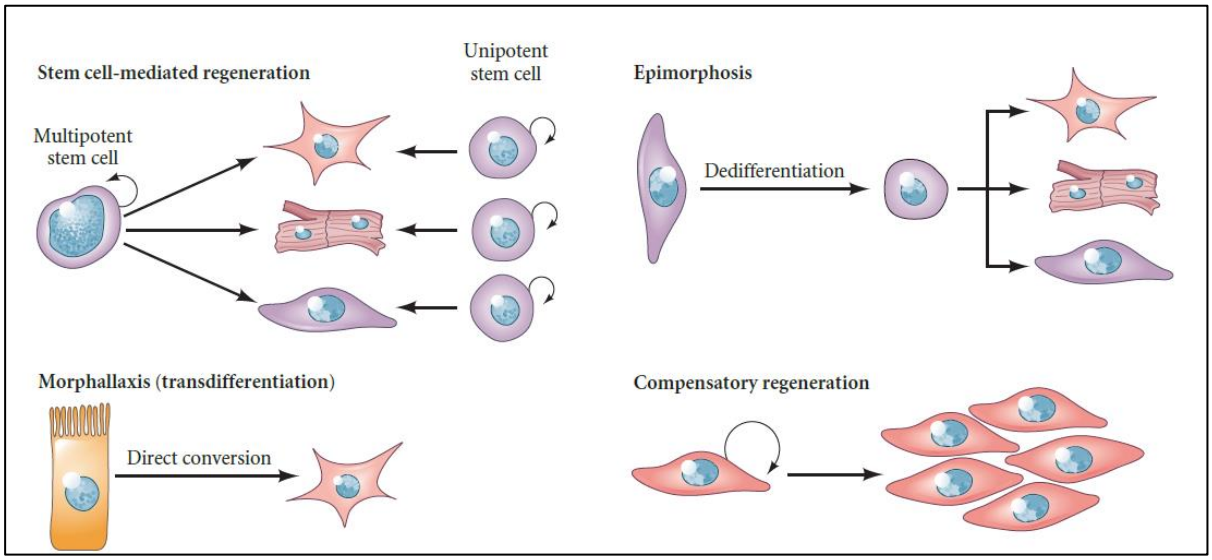

Figure 1.1: Types of Regeneration (Source: Gilbert, 11th edition, 2016)

4. Compensatory regeneration.

   In this type of regeneration the existing cells divide but maintain their differentiated form unlike observed in the process of epimorphic regeneration. The new cells arise from the division of the adult cells and not from the resident stem cells (Michalopoulos, 2017). This type of regeneration is observed in the liver of mammal (Figure 1.1 and 1.2).

Though these categories are presently accepted, planaria, another species possessing regenerative properties does not fall fully in either morphallaxis or epimorphic regeneration. Agata et al., (2007), based on the molecular and cellular studies have stated that these organisms, since do not fall under the current classification, there should be some modifications brought about in the classification system. According to their review, regeneration in all animals involves the formation of a distal structure, shortly after amputation, in a process called distalization, and this distal structure (Wound epidermis or Blastema) interacts with the tissue stump, to modify the positional information resulting in correct and complete restoration of the original structure. The cells which will form the new tissues may be provided by the stump directly (stem cells or transdifferentiating cells) or may first accumulate in a blastema as multipotent cells. Therefore, ‘distalization’ and ‘intercalation’ may well be the key concepts with which one may be able to explain all forms of regeneration (Agata et al., 2007).

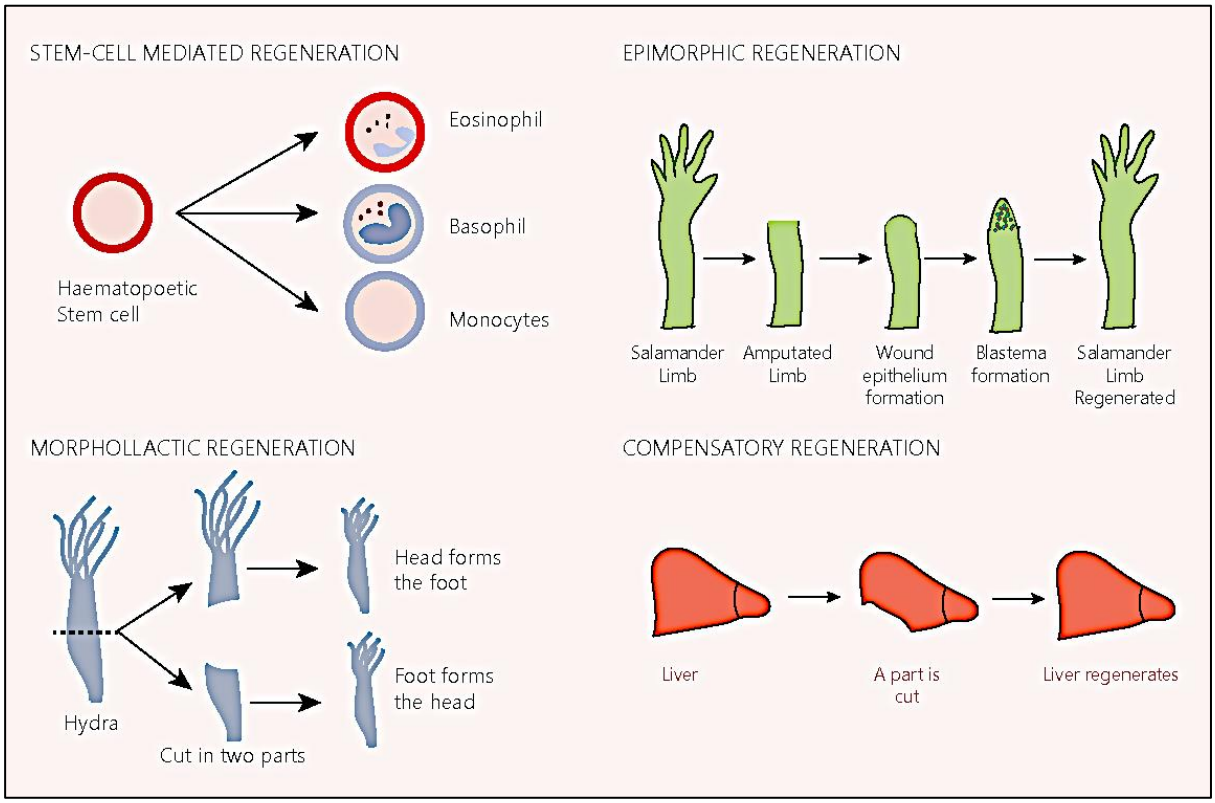

Figure 1.2: Examples for each of the modes of regeneration (Adapted from Gilbert, 11th edition, 2016)

MODEL ORGANISMS FOR THE REGENERATIVE STUDIES

Although regeneration takes place in nearly all species, several organisms have emerged as particularly interesting models for the study of regeneration (Figure 1.3). The near totality of hydra and planarian regeneration is unmatched. They are able to regenerate complete organisms following amputation or even complete individuals from very small fragments. Certain salamanders are unique among tetrapods in being able to regenerate whole limbs, and frog larvae are often used to study the regeneration of the tail and the lens of the eye. Zebrafish have recently proved advantageous for investigating the mechanisms of the central nervous system, retina, heart, liver, and fin regeneration. Although, mammals are unable to rebuild whole appendages, individual tissues and organs do possess variable regenerative capabilities; most notable are the antlers of deer.

The study on invertebrate regeneration is not new as it has been carried out for more than 200 years (Lenhoff et al., 1986). The diploblastic organism, *Hydra vulgaris* is one of the most studied invertebrates along with the triploblastic, bilaterally symmetrical freshwater planarian such as *Schmidtea mediterranea* and *Dugesia japonica* (Lenhoff et al., 1986). This species

does not get killed even with the loss of head, rather it can easily regenerate it. Hydra constantly keeps replacing its cells which may be lost during physiological turnover and hence one can even say they are immortal (Martínez, 1998). It is surprising yet true, within the initial hours of head decapitation, proliferation is not evident during regeneration (Holstein et al., 1991). Instead, reposition of the existing cells is observed along the remaining body, this displacement of cells will eventually reform the lost body part. The cells replacing the lost ones arrive from the gastric column, which undergo determination and are differentiated accordingly, to reform the missing part (Wolpert et al., 1971).

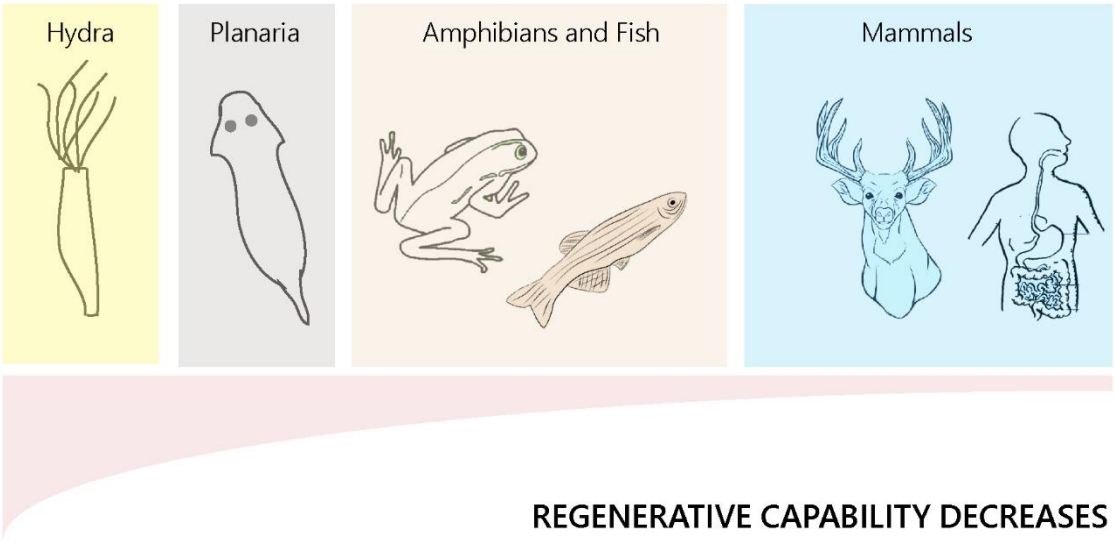

Figure 1.3: Representative organisms and their comparative regenerative capabilities (Adapted from Gilbert, 11th edition, 2016).

The planarians, which are free-living freshwater organisms, have been studied for more than 100 years and are a classical model of animal regeneration (Reddien and Alvarado, 2004) ever since, it is experiencing a renaissance in terms of regeneration research (Newmark and Alvarado, 2002; Reddien and Alvarado, 2004). Unlike hydra, which simply rearranges the existing cells, planarians regenerate missing body parts by first assembling a specialized structure known as the blastema, arising from the proliferation of pre-existing somatic stem cells known as neoblasts, further these neoblasts give rise to the lost part of the body.

Several vertebrate species have noteworthy regenerative capacities. Newts and salamanders are perhaps the most remarkable in this respect, followed by fish and then mammals, which by

comparison occupy a fairly distant third place. There are still no answers so as to why such a wide gamut of regenerative capacities exists in the vertebrates.

In general, fish have good regenerative capabilities, and the zebrafish is beginning to provide an excellent opportunity to study regeneration in lower vertebrates (Woods et al., 2005; Chávez et al., 2016). Zebrafish are easily reared in the laboratory, their developmental time is short and genetic screens have produced numerous mutants, including some that elicit defective regeneration. Since the genome is now mapped, microarray analyses are possible, and transgenesis and knock-down technology using morpholinos is readily available. Furthermore, chemical mutagenesis and small molecule screens have provided both developmental and regeneration mutants (Peterson et al., 2000).

Regeneration in amphibians is thought to be mediated mainly by extensive cellular transdifferentiation. Terminally differentiated cells at the site of amputation, dedifferentiate and then re-differentiate to form the lost part. In mammals, contrastingly, the transdifferentiation has been observed in only a few types of cells, such as the endothelial cells of the pancreas (Hao et al., 2006) and the Schwann cells of the peripheral nervous system (Harrisingh et al., 2004). Not very long back, the role of stem cells in the amphibian regeneration was also uncovered (Morrison et al., 2006), suggesting that regenerative capacities in these animals might involve both differentiated and undifferentiated cell types.

REGENERATION IN REPTILES

Many reptiles possess the ability to replace a lost tail through epimorphosis. Regeneration of 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 other amniotes. When we see the regenerative ability in lizards, they can restore nerve cells, part of a lower mandibular axon, and the entire tail (Simpson, 1965; Bryant and Bellairs 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 a specific region of the 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 bones (Alibardi, 2010) and secondly repair of dermal bones by the formation of osteoblasts, without involving secondary cartilage production (Irwin and Ferguson, 1986; Lozito et al., 2016). Regeneration is not observed in snakes except during moulting of skin (Maderson, 1971; Maderson et al., 1978; Smith and Barker, 1988; Chang et al., 2009; Klein and Gorb, 2012). 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; Vivien et al., 2016). Even so, reptiles as models of regeneration have been completely underestimated and neglected for unclear reasons.

REGENERATION IN LIZARDS

Lizards are one of the largest and the most diverse groups of terrestrial vertebrates. Till date, more than 6100 species are recognized (Uetz and Hošek, 2016; compared with∼5400 mammals). These species encompass a wide range of morphologies that include species which are limbless, animals whose body sizes ranges in adult from ∼1.6 to >300 cm (Hedges and Thomas, 2001; Laver et al., 2012) and locomotory behaviours which includes crawling, running, burrowing and swimming. Lizards (eg. Geckos) are the closest group of organisms that have an ability to replace a lost body part, in terms of evolutionary hierarchy to mammals. 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, making reptiles an attractive model to study tissue and organ regeneration. Lizards, albeit limited, have ability to regenerate the lost part is more like their vertebrate ancestors namely fish and amphibian. However, lizards replace their lost appendage (tail) with an unsegmented tail quite contrary to the metamerically segmented original tail. Nonetheless, this replacement of tail is good enough for the animal to regain its social acceptability and survival. Hence, though neglected, lizards provide an excellent platform to answer the questions related to the regeneration biology. One of the complex tissue that a lizard can regenerate is the tail following amputation.

Northern house gecko *Hemidactylus flaviviridis* from Gekkonidae - a family having 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 18-20 cm

in length, the body is covered with small keeled scales, showing distinct variation in pigmentation, leading to striking variations in body colour. At night, it is typically greyish, olive-brown in colour with indistinct bands on the back while at during day time it is usually much darker in colour with chevron-shaped bands. Body and the head are usually flat, and the tail has enlarged tubercles (wart-like bumps and ridges) along the dorsal side (Nanhoe and Ouboter, 1987; Halliday and Adler, 2002; Bartlett and Bartlett, 2006; Gardner et al., 2007). Their toes possess broad pads which are covered with small scales called scansors, each of it has up to 1,50,000 microscopic, highly branched, hair-like structures, known as setae, and at the toe tips, small claws are present (Halliday and Adler, 2002; While et al., 2019).

H. flaviviridis has been used in our department to study the mechanisms of regeneration for 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; Sharma and Suresh., 2008; Sharma et al., 2011; Yadav et al., 2012; Pillai et al., 2013; Buch et al., 2017, 2018; Murawala et al., 2018). *H. flaviviridis* can autotomize its tail like many other lizard species. Tail autotomy is normally seen when the animal is trying to get away from the prey, in other words escaping predation or similar threats. It is an indeed voluntary action and losing tail has its advantages and disadvantages both. Loss of tail not only causes heavy physiological drawback, but also leads to survival, which is the main aim of the animal. The success of autotomy, as a phenomenon in the animal kingdom can be testified by the fact that it is exhibited by diverse groups from crustaceans to chordates, including echinoderms, amphibians and reptiles (Juanes and Smith, 1995; Bernardo and Agosta, 2005; He et al., 2016; Cooney et al., 2017). As analysed by McConnachie and Whiting in 2003, lizards form 13 of the 20 families possess this ability of autotomy. The two main advantages of caudal autotomy are (i) Escape: Tailless lizards have a higher likelihood of being captured than their tailed counterparts, since autotomy helps in escape even after capture, in many cases. (ii) Distraction: In many of the species, the lizard tail moves randomly and rather violently after autotomy, serving to distract the predator and helping the lizard to flee.

In order to reduce the tissue damage and to facilitate autotomy, lizards along with tuatara, amphibians and even some snakes have evolved to possess fracture planes (Arnold, 1984; Bellairs and Bryant, 1985; Clause and Capaldi, 2006; Maginis, 2006; Gilbert et al., 2013).

They are nothing but connective tissue partitions that pass transversely between segments of the dermis, muscle and adipose tissue, subdividing individual tail vertebra into cranial and caudal components (Figure 1.4) (McLean and Vickaryous, 2011; Sanggaard et al., 2012; Lozito and Tuan, 2017). The fracture plane is split and the intervening vertebra is broken during the intravertebral form of autotomy. In order to minimize the blood loss, thick smooth muscle sphincters located on the major arterial supply i.e. the caudal artery to the tail constricts. This constriction is brought about immediately after the tail is released, the sphincter proximal to the site of tail loss contracts which ultimately leads to a reduction in blood loss (McLean and Vickaryous, 2011). Tail autotomy can occur several times, provided the original tail still has the fracture planes. Hence, the organism is capable of losing its tail more than once. The tail is required for locomotion, social and sexual interaction. Apart from these functions, the tail is the main site for storage of energy (fat). As much as 40 % of the body weight could be made up by the tail which carries energy reserves in the form of fat (reviewed by Clause and Capaldi, 2006). It is therefore too valuable an organ to be lost and regeneration becomes a crucial event following autotomy.

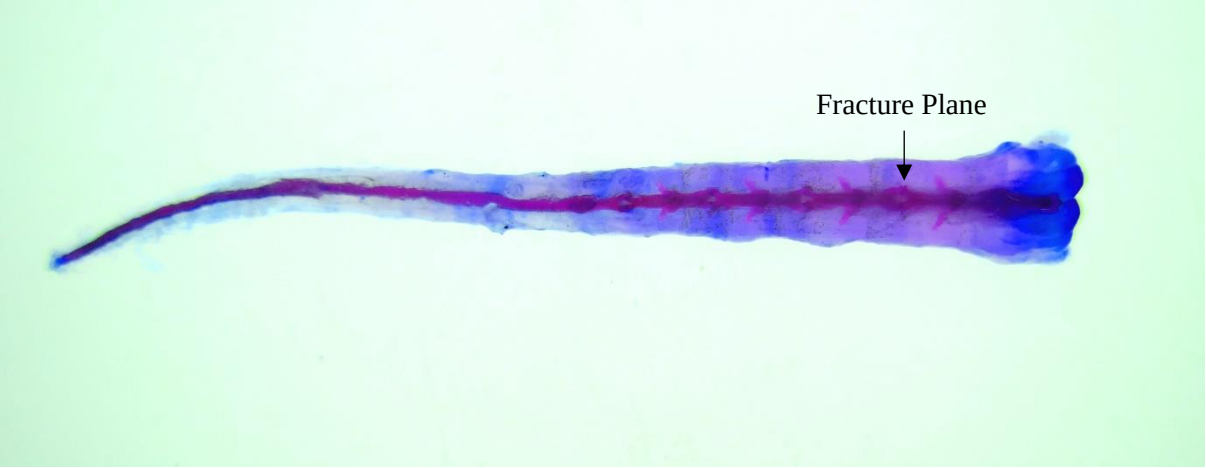

Figure 1.4: Lizard tail depicting the fracture plane. The tail is stained with alcian blue-alizarin red stain.

THE PROCESS OF EPIMORPHIC REGENERATION

Epimorphosis in vertebrates shares some key similarities with development in the embryonic stages, however, the two are not entirely the same, as shown in numerous studies at the molecular and genetic levels carried out over the years. The pattern of expression of some genes during regeneration is different from that in development (Bryant et al., 2004). As categorized by Carlson et al. (1998), appendage regeneration in vertebrates proceeds via three major stages:

Wound Healing, Blastema formation and Growth and Differentiation. The first two stages can be considered as the *preparatory phase* of regeneration. These distinguish regeneration from development. The third stage is the *redevelopment phase*, which is largely similar to development. The preparatory phase is inevitably essential for regeneration to occur.

1. Wound Healing: After amputation or injury, vertebrates capable of epimorphosis quickly undergo wound healing. This comprises of a short inflammatory phase with immune molecules accumulating locally to prevent infection and also to counter the damage which was caused by the injury. This immune response however does not persist for long. The exposed mesenchymal tissue is rapidly covered with migrating epithelial cells from the circumference of the intact epidermis (Bryant et al., 2004 and Yokoyama, 2008). The main function of this epithelial covering is to provide a favourable environment to the underlying mesenchyme so that it can advance through the stages of regeneration while eliminating the risk of infection. The covering, called the *wound epidermis*, is initially a single layer, which, in a short while, thickens to form a multi-layered structure called the *apical epithelial cap* (AEC). This first stage of regeneration is called as wound epithelium. Signals from the nerve terminus have an important part in regeneration. In salamanders, they are known to prevent skin formation over the AEC and therefore allow its transition to further stages of regeneration (Bryant et al., 2004). During wound healing, an array of proteins is secreted locally. Matrixmetalloproteinases (MMPs) are crucial for facilitating the migration and proliferation of epithelial cells over the wound site. Other vital factors appearing at this stage include members of the Fibroblast Growth Factor (FGF) pathway and the Transforming Growth Factor-β (TGF-β) pathway. These factors, among some others, so as to say, activate tissues of the stump, leading them to form the blastema.

2. Blastema formation: As the AEC matures and the damaged or dead cells are cleared from the amputation site, there occur significant changes in the tissues at the amputation plane. Molecular signals from the AEC stimulate these changes, leading to the formation of a pool of undifferentiated cells, which will give rise to the new appendage. This cell mass, called the blastema, is characteristic of epimorphic regeneration. These cells are contributed by stump tissue by either dedifferentiation of mature tissue or by activation of resident stem cells or, as often seen, both (Stocum, 1999; Santos-Ruiz et al., 2002). However, spatial and temporal patterns of gene expression in the blastema stage are not the same as that in the blastula during development (Bryant et al., 2004). Blastema formation is nerve-dependent.

Cells herein are fast-proliferating and the structure quickly enlarges into a cone. Blood vessels are among the earliest differentiated tissues to invade the blastema and allow it to grow further and proceed to the redevelopment phase of regeneration.

3. Growth and Differentiation: The third and longest-running stage of epimorphosis involves continued proliferation of blastemal cells with simultaneous differentiation into varied tissues that will form the new appendage. Patterns of gene expression are similar to those during development (Carlson et al., 1998). Regeneration ends when the full size of the appendage has been re-formed. The process of epimorphic regeneration of appendages in the animal model used in the current study – lizard *H. flaviviridis* – is described below.

SCAR-FREE WOUND HEALING AND REGENERATION OF TAIL IN LIZARD

Observations in our lab suggest that tail regeneration in the lizard *H. flaviviridis* follows the above-stated steps without any deviation (Figure 1.5 A). Immediately after amputation, epidermal cells from the circumference of the tail migrate to cover the wound surface. This wound-healing phase is achieved strictly through cell movement without cell division and is completed within 48 h after amputation. Over the next few days, this thin layer of epithelial cells thickens into the multilayered AEC by 4 days post amputation (dpa) and the stage is called as wound epithelium. The thickening of the epidermis is accompanied by histolysis of stump tissues such as bone and muscle, from which emerges dedifferentiated cells, that accumulate directly beneath the thickened AEC. These cells re-enter the cell cycle and give rise to the blastema, an accumulation of mesenchyme-derived cells that are believed to be largely, if not completely, originating from dedifferentiation of previously differentiated cells by 6 dpa. At the early bud stage, the blastema is visible only as a small protuberance, however, continued cell division results in its enlargement into a cone-stage blastema. As the blastema continues to expand, the dedifferentiated cells re-differentiate into tail tissues (9 dpa) following many of the same patterning programs that were originally employed during embryonic tail development.

Therefore, following these three stages of wound healing, the tail can regenerate itself however the limb cannot. In order to understand why the limb cannot regenerate, examining how the wound heals is necessary. Limb heals in such a way that a scar is formed near the wound which resembles the mammalian wound healing also known as scarred wound healing.

WOUND HEALING IN MAMMALS

The response to any injury in case of mammals follows four overlapping stages namely, hemostasis, inflammation, granulation and scarring. The first stage of wound repair, hemostasis occurs immediately after the tissue damage, in order to restrict blood loss. Once the blood clot forms, inflammation succeeds it wherein the inflammatory pathway and immune system get activated. This is required to remove the dead and devitalised tissues and also to prevent infection caused by the entry of the pathogen. A platelet plug is formed by a fibrin matrix initially at the wound site which becomes a scaffold for the infiltrating cells. Once the platelet plug is formed, neutrophils are then recruited to the wound site where they clear the degranulated platelets and the products of bacterial degradation (Grose and Werner, 2004; Barrientos et al., 2008). Following neutrophils, monocytes appear at the wound site which later on differentiates into macrophages which are thought to be important during the latter events post-injury, nonetheless importance of neutrophils and macrophage are not well understood in the healing process. Although few studies have shown that a deficiency in either of cell type can be compensated by the other one during the inflammatory response (Martin and Leibovich, 2005). In the absence of both cell types scarring is seen to be reduced (Martin et al., 2003). Inflammation persists for a long time followed by new tissue formation which is underlined by proliferation and migration of various cell types.

In mammalian repair, keratinocytes first migrate over the injured dermis which is marked as the first event in wound healing. Following the keratinocytes covering the wound, new blood vessel forms and this process is known as angiogenesis. The sprouts of capillaries are associated with fibroblast and macrophages which replace the fibrin matrix, a granulation tissue. This granulation tissue later acts as a substratum for the migrated keratinocytes during the repair process. The keratinocytes that are behind the leading edge proliferate and mature and, finally, restore the barrier function of the epithelium. Angiogenesis can also result from the recruitment of bone-marrow-derived endothelial progenitor cells, although the magnitude of this contribution is small, at least in non-ischaemic wounds (in which the concentration of oxygen is normal) (Bluff et al., 2007; Inglis et al., 2016). Now, the fibroblast which is attracted from the wound site or even from the bone marrow are stimulated by the macrophages which lead to differentiation of these cells into myofibroblast (Opalenik and Davidson, 2005; Manning et al., 2015; Minutti et al., 2017). Myofibroblasts are contractile cells that, over time,

bring the edges of a wound together. Extracellular matrix in the form of collagen is secreted by the interaction of fibroblast and myofibroblast, which ultimately leads to the formation of scar (Werner et al., 2007). The last stage of wound repair is the remodelling phase which begins after a few days of granulation tissue forms. The scar tissue maturation occurs during which all of the processes activated post-injury, cease. Mostly endothelial cells, myofibroblast, and macrophages are seen to undergo apoptosis (programmed cell death), or even exit from the wound site which leaves the extracellular matrix consisting of collagen fibres and other ECM proteins (Szabowski et al., 2000; Darby et al., 2016). Additionally, over the next few months, the acellular matrix is actively remodelled wherein type III collagen is present as a backbone along with the predominant presence of type I collagen (Lovvorn et al., 1999). This remodelling process is mainly carried out by matrix metalloproteinase (MMPs) which digests the extracellular matrix, and also strengthens the repaired tissue. Nonetheless, the repaired site never regains the original property or function since the healed structure has a big chunk of scarred tissue (Levenson et al., 1965; Darby et al., 2016). It is very interesting that the other vertebrates such as zebrafish, amphibians, do not produce either of these collagens, instead they secrete type VI and type XVIII collagens (Rubin et al., 2000). This finding suggests a degree of evolutionary plasticity that is not observed in the earlier stages of wound repair.

WOUND HEALING IN LIZARD LIMB (SCARRED WOUND HEALING)

The above said observations were seen even in lizard limb upon amputation. All the four stages which occur during the mammalian wound healing are reported during the limb healing of lizards. In Figure 1.5 B, a trend observed during *H. flaviviridis* limb healing has been portrayed from the various studies performed in our lab. When the limb is amputated, a lot of blood loss occurs unlike in tail. Blood clot formation takes a longer time and is visible only by three days post-amputation. The epidermal covering is not as quick as observed in tail rather it resembles the mammalian wound healing and hence it can be observed by 6 dpa. The repaired tissue has a lot of collagen content which is contradictory to tail wound healing. This collagen deposition eventually leads to scar formation by 9 dpa. Once the scar forms, maturation takes a long time like detected in the skin wound healing as mentioned previously.

Hence, from this it can be said that the lizard tail undergoes scar-free wound healing which leads to restoration of the lost part whereas the limb follows a scarred wound healing mechanism which inhibits the organ restoration. Further, wound healing, is an incredibly

complex biological process with intricate molecular interaction amongst various cells at the site of injury, is modulated by the timed expression of a myriad of regulatory factors.

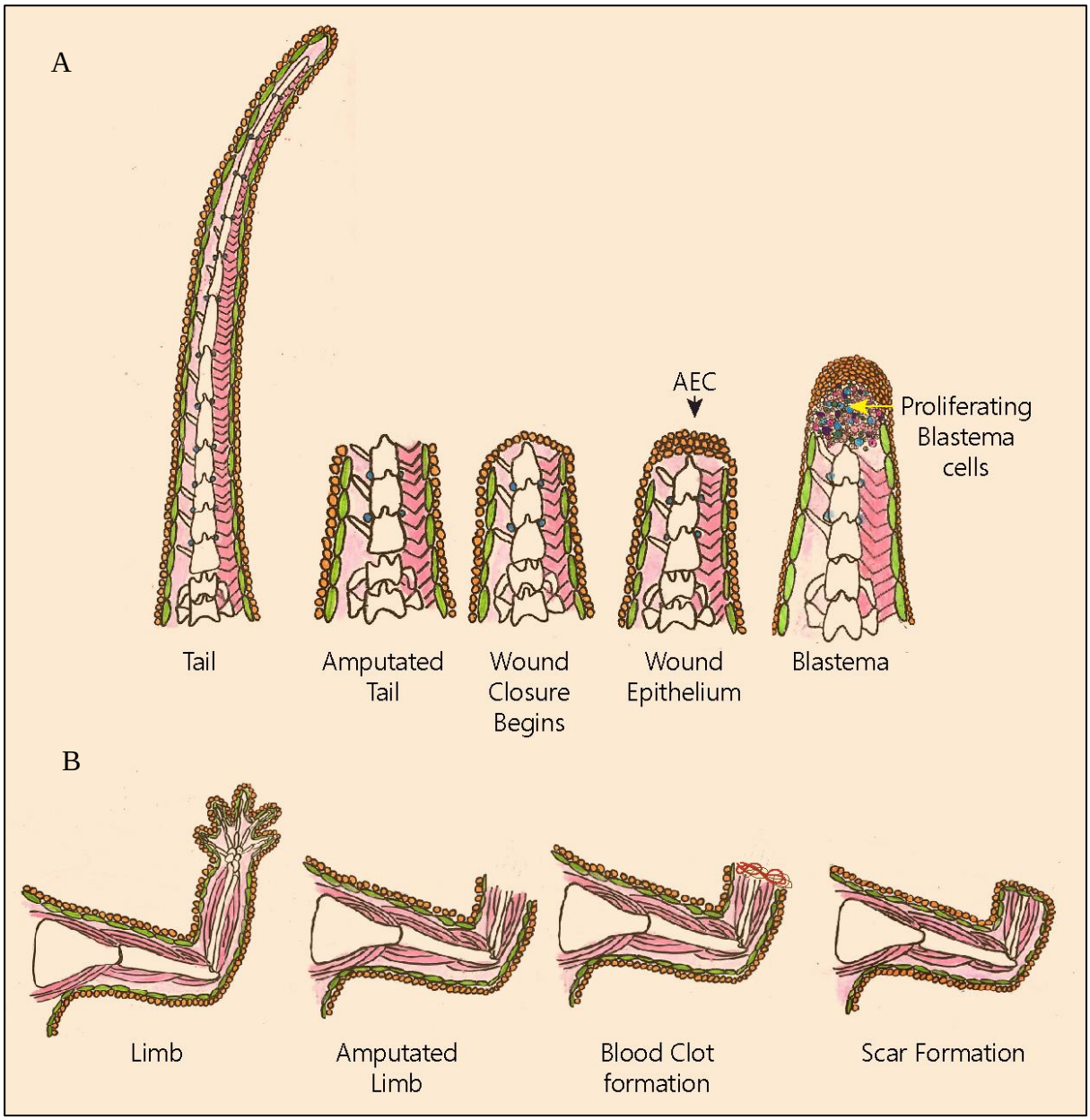

Figure 1.5: Stages of wound healing in the regenerating tail and non-regenerating limb following amputation

Important among these factors are the members of epidermal growth factor (EGF), transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), interleukin (IL) and tumour necrosis factor-α (TNF-α) families (Penn et al., 2012; Makanae et al., 2016). In addition, vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF), platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF) are known to influence the process of wound healing (Barrientos et al., 2008;

Matsumoto and Ema, 2014). The main pathway targeted for the current study was FGF, along with which other associated factors involved in regeneration and wound healing were studied in depth.

FIBROBLAST GROWTH FACTORS AND THEIR RECEPTORS

Fibroblast growth factor (FGF) was first identified around 46 years ago that displayed mitogenic activity in the pituitary extracts (Armelin 1973; Gospodarowicz 1974). Eventually observations made in this experiment unravelled a large family of growth factors that directly or indirectly had an effect on cell proliferation, differentiation, motility and survival (Vlodavsky et al., 1990; Ornitz and Itoh, 2001; Beenken et al., 2012; Suh et al., 2018). There are ample of evidences where role of FGF family in development and embryogenesis has been vital. Hence, it is safe to say that FGF, along with other signalling molecules like WNT, Sonic hedgehog (SHH) and bone morphogenetic protein (BMP) regulate or induce the development of most organs in the vertebrate body. FGFs consist of a family of twenty-three members (FGF1 to FGF23) (Figure 1.6), each consisting of a conserved core region of about 155 amino acids (Itoh and Ornitz, 2004; Brewer et al., 2016). FGFs are known to have a multifunctional role with a wide variety of effects, mitogenesis being the most important one, however, they have regulatory and endocrine effects too. FGFs are required not only during mitosis but are required for many other processes and hence, are referred as pluripotent and even promiscuous growth factors due to their role in several cell types (Vlodavsky et al., 1990; Ornitz and Itoh, 2001; Beenken et al., 2012; Suh et al., 2018).

The signal transduction occurs via high-affinity transmembrane protein tyrosine kinases, FGF receptors. There are currently 5 major FGFR from FGFR1-5, which have different affinities for various receptors given in the following Table 1.1 (Katoh, 2016). Variation in the receptor is brought about by the alternative splicing in the extracellular domain of FGFR1-3 which is the major reason why it affects the affinity of the FGFs to bind to the receptors. FGF1 and FGF2 bind to all the receptors while FGF7 has affinity towards FGFR2, designated FGFR2IIIB (Ornitz et al., 1996). A characteristic attribute of FGFs is how they interact with heparin or even heparan sulphate proteoglycan. This interaction stabilizes FGFs to thermal denaturation and proteolysis leading to limited diffusibility of these FGFs. Moreover, this interaction is essential as it leads to activation of the signalling receptors (Ornitz, 2000).

Table 1.1: List of FGFs and their affinities towards different FGFRs

RECEPTOR	LIGAND
FGFR1	FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF8, FGF10 and FGF17
FGFR2	FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF7, FGF8, FGF9, FGF10 and FGF17
FGFR3	FGF1, FGF2, FGF4, FGF8 and FGF9
FGFR4	FGF1, FGF2, FGF4, FGF6, FGF8 and FGF9
FGFR5	FGF1 and FGF2

ROLE OF FGF IN DEVELOPMENT AND REGENERATION

FGFs play a crucial role in the developmental processes like mesoderm induction, anterior-posterior patterning, limb formation, brain development and angiogenesis. A table with various FGFs and their role in development has been summarized (Table 1.2). The processes like formation of apical ectodermal ridge (AER), mesenchyme proliferation, angiogenesis, neurogenesis and differentiation are mostly regulated by various FGFs. Irregularities in their function lead to developmental defects in vertebrates as well as in invertebrates. FGFs also stimulate cells to migrate chemotactically towards them (Landgren et al., 1998; Howard et al., 2010; Zhang et al., 2015). This is of importance both in angiogenesis and in wound healing (Burgess and Maciag, 1989; Powers et al., 2000). Further, FGFs stimulate cells to secrete proteases such as plasminogen activator (Miralles et al., 1998; Cauwe et al., 2007), collagenase (Ornitz and Marie, 2002; Schlutz et al., 2005) and gelatinase (Weston and Weeks, 1996; Honnegowda et al., 2015). Together, these FGF-stimulated cellular functions, *viz*. cell proliferation, migration, and protease secretion, provide the basis for matrix reorganization and angiogenesis which are important physiological functions of FGFs (Figure 1.7). FGFs also influence cell differentiation, stimulating the process in some cell types (Robinson et al., 1995; Klint et al., 1999) while inhibiting it in others (Tirosh-Finkel et al., 2010). Moreover, FGFs can also protect cells from undergoing apoptosis (Zheng et al., 2016; Tomida et al., 2017). Amongst all FGFs, FGF7 somehow has specificity for epithelial cell proliferation and hence, is also known as keratinocyte growth factor (Werner, 1998). Apart from having the ability to induce cell proliferation, FGFs can also regulate the processes like migration and differentiation of their target cells and some FGFs have also shown to have cytoprotective role which supports cell survival under immense stress condition (Basilico and Moscatelli, 1992; Werner, 1998;

Ornitz and Itoh, 2001). FGFs have been identified in the limb development as well, dominantly during the AER formation. AER is very important for the positioning of the limb outgrowth and also for determining the size of the limb. For instance if the AER is rotated by 90 degree then the limb grows at right angle when compared to the original axis (Foster et al., 1999). Of the 23 known FGF genes, 5 are expressed in the distal part of the established limb bud, 4 in the AER (Fgf2, Fgf4, Fgf8, Fgf9), and 2 are detected in the underlying mesenchymal tissue (Fgf2 and Fgf10), and their role is apparently to provide AER function, either directly or indirectly.

Table 1.2: FGFs and their role in the process of development

Ligand	**Role**
FGF1	Endothelial cell proliferation and angiogenesis
FGF2	Endothelial cell proliferation and haematopoiesis
FGF3	Chondrogenesis and negative regulator of bone growth factor during ossification
FGF4	Formation of AER, mesenchyme proliferation, angiogenesis and neurogenesis
FGF5	Cell proliferation and differentiation
FGF6	Embryogenesis
FGF7	Regulator of liver progenitor cells
FGF8	Cell growth, morphogenesis and tissue repair
FGF9	Proliferation of mesenchymal cells
FGF10	Formation of AER
FGF11	Proliferation
FGF12	Inhibition of apoptosis
FGF13	Neural development
FGF14	Voltage-gated sodium channels activity in neuron
FGF16	Embryonic heart development
FGF17	Embryonic development
FGF18	Chondrogenesis and osteogenesis
FGF19	Skin and cartilage formation
FGF20	Wound Healing
FGF21	Blastema formation
FGF22	Hair development
FGF23	Vitamin D metabolism

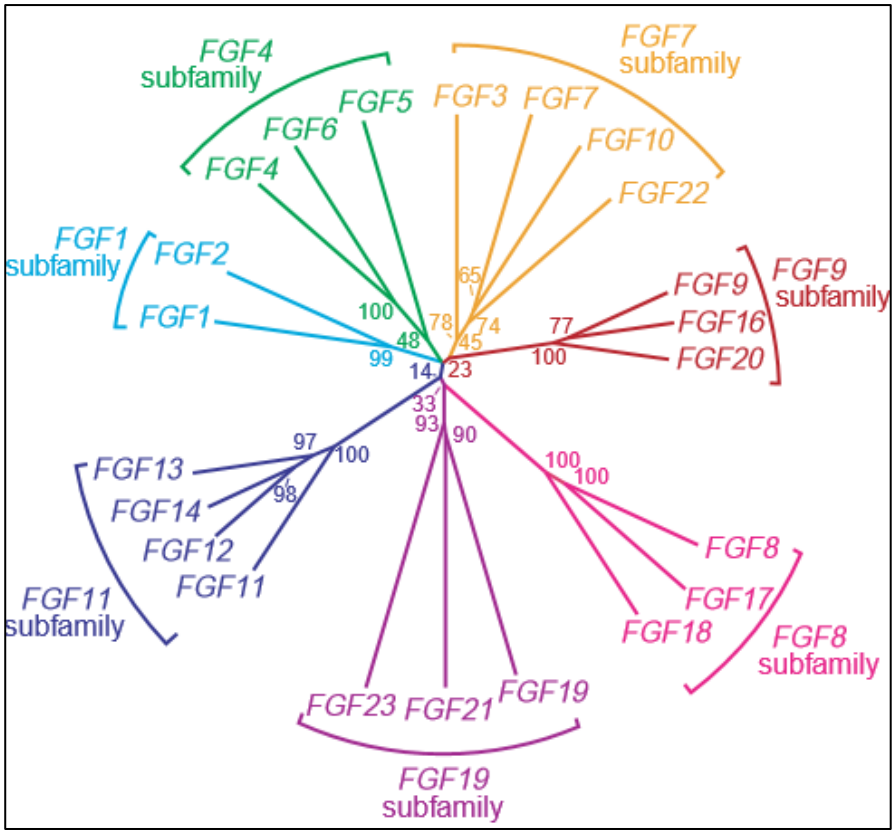

Figure 1.6: The evolutionary relationships within the human fibroblast growth factor (FGF) gene family. Twenty-two FGF encoding genes have been identified in the human genome. (Source: Itoh and Ornitz, 2004)

Role of FGF in wound repair have been reported in numerous *in vivo* studies thus drawing our attention to investigate their role in lizards tail regeneration. FGF1 and FGF2 in particular, have shown to stimulate angiogenesis in various *in vivo* and *in vitro* systems (Risau, 1990; Marwa et al., 2016). Amongst other cells, fibroblast and keratinocytes are also induced by FGFs to undergo proliferation at the wound site (Werner et al., 2007). Thus FGFs are clear candidates when it comes to contributing towards the wound healing process and this hypothesis has been supported by many studies wherein local application of FGF1, FGF2, FGF4, FGF7 or FGF10 have stimulated the process of tissue repair (Werner et al., 2007; Fumitaka et al., 2019).

Not surprisingly, FGFs are known to play significant roles in epimorphic regeneration as well. During wound epithelium and blastema formation, in both urodeles and larval anurans FGF8 begins expressing (Christen and Slack, 1997; Han et al., 2001; Christensen et al., 2002). FGF8 and FGF10 expression correlates with regenerative capacity in *Xenopus*, amputation at a later, non-regenerative stage of development, fails to result in the formation of a blastema or expression of either of these FGF genes (Yokoyama et al., 2000). Importantly, treatment of a

non-regenerative stage *Xenopus* limb stump after amputation with FGF8-soaked beads results in partial regeneration, and treatment with FGF10 stimulates expression of several genes that are expressed in regenerating limbs, including shh and msx1 resulting in significant regeneration (Yokoyama et al., 2001). Similar studies in the chick, where amputation of the limb bud always results in regeneration failure (no matter what stage), show that treatment of the amputation surface with FGF2 or FGF4 induces a regenerative response (Taylor et al., 1994; Makanae and Satoh, 2018). FGF1 is also known to influence blastemal cell proliferation during amphibian limb regeneration (Satoh et al., 2018; Saito et al., 2019). Fischer and co-workers (2003) have demonstrated that FGF is absolutely necessary during caudal fin regeneration wherein pre-blastemal mesenchymal cells express FGFR1 along with high expression of FGF24 (wFGF). There are several studies depicting FGF signalling being an integral pathway during fin regeneration (Poss et al., 2000; Wills et al., 2008; Shibata et al., 2016). Additionally as reported by Whitehead et al. (2005) temperature-sensitive mutants of FGF20a does not promote the blastema formation in the caudal fin of zebrafish, depicting FGF20a being an important ligand required for regeneration. These studies mentioned above clearly indicate that FGFs play a crucial role in mediating the blastema formation during regeneration, and hence, they would directly be related to the processes required for the same. New cells come near the site of injury and proliferate to give rise to a mass of undifferentiated cells know as blastema (Patel et al., 2019; Tsai, 2019). This blastema is formed by either de-differentiation of the existing cells and proliferation of a resident stem cell population or sometimes even by the division of satellite cells (Murawala et al., 2018). But the extent of each of this mode used depends on the species or even in the same species it depends on the type of tissue undergoing regeneration and hence, cannot be generalized. A third type of mode which leads to addition of cells in the blastemal pool is now being accepted known as epithelial to mesenchymal transition (Alibardi, 2012; Subramaniam, 2016; Abnave et al., 2017).

Cell transition from epithelial to mesenchymal type is a very common phenomenon observed during the embryonic development. This transition is highly plastic and a dynamic process. This shift from the epithelial to mesenchymal population is commonly called as epithelial to mesenchymal transition (EMT). During EMT the epithelial cells lose their characteristics like expression of the adhesion molecules and rather become invasive in nature. These so-called mesenchymal cells can also transition back to the epithelial type by a reverse process called as mesenchymal to epithelial transition.

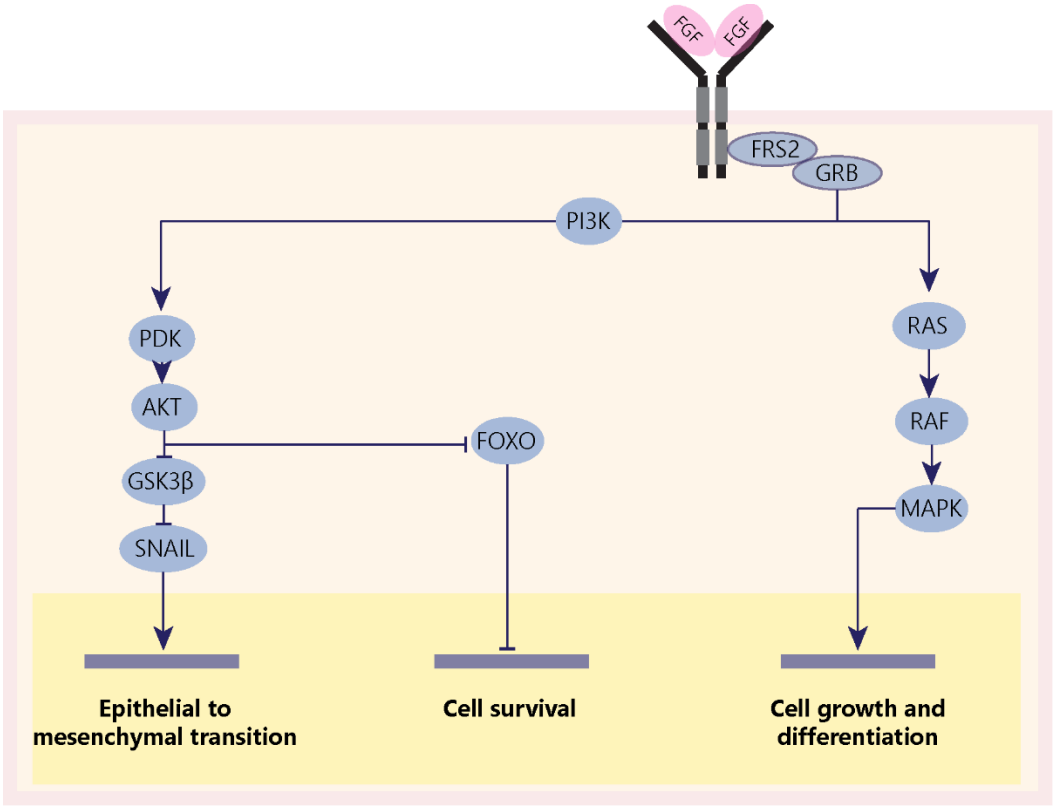

Figure 1.7: FGF signalling pathway (Adapted from Katoh and Katoh, 2006)

During this kind of a transition the cells start expressing the adhesive molecules again and adopt the apicobasal polarization, key feature of the epithelial tissue. (Wu et al., 1996; Thiery et al., 2009). Now there are a few reports which suggest that the blastema formation which takes place during regeneration might be a result of EMT (Alibardi 2009). Blastema formation is known to occur through the resident stem cells which undergo proliferation and by the dedifferentiation of the existing cells. However, since developmental processes get activated during regeneration, EMT being one of them, is also a switched on. In lizard tail wound healing, there are few reports for EMT playing a role in contributing towards the blastema formation. Regulation of EMT in the adult tissue is mediated mainly through extracellular matrix components, FGFs, epidermal growth factors (EGF), cytokines and transforming growth factors (TGF-β) (Thiery and Sleeman, 2006; Acloque et al., 2009). Amongst these, TGF-β and FGF are considered as key mediators of EMT and are frequently and abundantly expressed in various tumours. TGF-β signalling not only contributes to EMT during embryonic development, but also induces EMT during cancer progression in oncogenic cells (Moustakas and Heldin, 2007). TGF-β activates Smad proteins, which further transcriptionally regulate several genes including δEF1, Snail, Twist, HMGA2, and Ids, that lead to EMT particularly through the transcriptional repression of E-cadherin (Zavadil and Bottinger, 2005; Horiguchi

et al., 2009). Among 23 FGFs, FGF-2 and FGF-4 are key regulators of EMT during development and cancer progression (Strutz et al., 2002; Strutz and Neilson, 2003). However, it has not been determined as how FGF2 transmits signals to induce EMT and promote cancer progression since epithelial cells dominantly express the isoform FGFRIIIb, which typically do not have affinity for FGF2. Few years back there was a report showing cancer cells overexpressing the FGFRIIIc isoform, however the reason behind how the epithelial cells along with cancer cells upregulates the expression of this particular isoform is unknown (Acevedo et al., 2007). As mentioned earlier the EMT pathway is promoted by many soluble growth factors, including FGFs (Savagner et al., 1994; Katoh and Nakagama, 2014). The activation of the FGFR/ERK pathway by FGF2 is required for promoting cell growth and even in activating EMT in the chordoma (Hu et al., 2014) while FGF10 can induce EMT of breast cancer cells (Figure 1.7) (Abolhassani et al., 2014). Furthermore, FGF8 can induce a more aggressive phenotype displaying EMT and enhanced invasion and growth in colorectal cancer cells (Liu et al., 2015a). From these studies one can expect FGF to be involved in the process of EMT during the blastema formation. It would also be interesting to check whether during the limb healing if at all, EMT gets switched on.

AIM OF THE STUDY

From the above information it is not farfetched to say that very few studies are available regarding the wound healing pattern of tail and limb in lizard. However, understanding the finer mechanisms that govern both the types of healing in lizard, can help us developing targeted methods to improve mammalian wound healing and reduce scarring. Since much data is not available for the growth factors which regulate the wound healing in lizard, our approach was to first identify these factors by doing a comparative proteome profiling followed by understanding the role of various FGFs in shaping the healing process. For achieving this, the study was divided into three main domains listed below.

1. Making a comparative proteome profile for the healing tail and limb by using 2-Dimensional gel electrophoresis technique.
2. Exploring the role of FGF and other key molecules in the proliferation, apoptosis and angiogenesis during the wound healing process
3. Investigating the expression of FGFs and other peptides that differentially regulate the process of EMT during scar-free and scarred wound healing.