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Introduction

Regeneration, by the simplest of definitions, is the reactivation of development in later life to restore missing tissues (Gilbert, 2014). It is a fundamental feature of all living organisms. However, when one places the various living beings on a scale of regenerative ability, one finds all living forms scattered across, from some with remarkable abilities to restore entire bodies from just small parts, to others capable of only reparative regeneration. It is needless to mention that we humans fall on the wrong side of this scale. One may presume, or perhaps logically argue that humans (and other closely related organisms) traded their regenerative capabilities for a number of other beneficial traits during the course of evolution. Even so, man, in his eternal quest to improve the quality of his life, has tried to gain an insight into the process of regeneration with a hope to employ it to his gain.

HISTORY OF REGENERATION RESEARCH

The study of regeneration dates back to as long as the 18th century when Abraham Trembley, René-Antoine Ferchault de Réaumur and Lazzaro Spallanzani observed and described the regenerative capacity of various animals. Trembley made all his observations on regeneration with a small pair of scissors and hydra held in the palm of his hand. It is intriguing that his simple methodology, all carried out without a laboratory set-up, set the standards for experimental repetition, thorough investigation and keen observational practices. And yet, a much larger contribution made by his work was in the dispute between the preformationists and the materialists. Trembley's observations gave strong support to the theory of epigenesis, helping to quash the theory of preformation (Sunderland, 2010).

The work carried out by these early regeneration scientists turned out to be the foundation for experimental biology, as also for scientific discussions and extrapolation based on experimental data. It is not very surprising that two centuries later, we still do not have as much knowledge about the process of regeneration itself, as one might expect. This can be said, given the overwhelming complexity of the process and the enormous number of 'factors' involved in it.

Research in regeneration had to wait for highly sophisticated techniques to be developed, and the observations, that had been made so long ago from now, have only recently begun to be addressed in detail. It is however surprising that the classification of regeneration, based on its mechanisms, has seen little change over all these years. Perhaps this, in some way, reflects the slow progress of research in this field.

TYPES OF REGENERATION

As more and more regenerating systems became known to the scientific community, it was realised that the mechanism is not entirely common among all of them. 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 the 19^{th} century and is mainly based on morphological evidence. Textbooks retain this classification even today and their description is still based largely on morphological differences among the two.

In morphallactic regeneration, the stump (part of the body left behind after amputation) undergoes remodelling such that the existing tissues reform the entire body. This type of regeneration therefore involves very limited cell proliferation and relies primarily on rearrangement of cells to take up all the functions of the body. As a result, it is associated with a reduction in the size of the organism. The best known and most widely studied example of morphallactic regeneration is the one exhibited by *Hydra*. When the *Hydra* body is cut into smaller pieces, each piece remodels itself to form the full body albeit smaller. Each of these newly formed organisms will eventually feed and grow to their full size (Figure 1A).

Epimorphosis, on the other hand, proceeds through the formation a regeneration blastema, which is assigned the role of forming the lost part of the organism. Thus, in this type, the stump tissue remains largely unchanged. It however plays the role of providing cells to the blastema for forming the new tissues (Figure 1B). This type of ‘add on’ (as expressed by Suzuki et al. in 2006) regeneration is seen in echinoderms and most vertebrates capable of large scale regeneration including teleost fish, salamanders and some lizards.

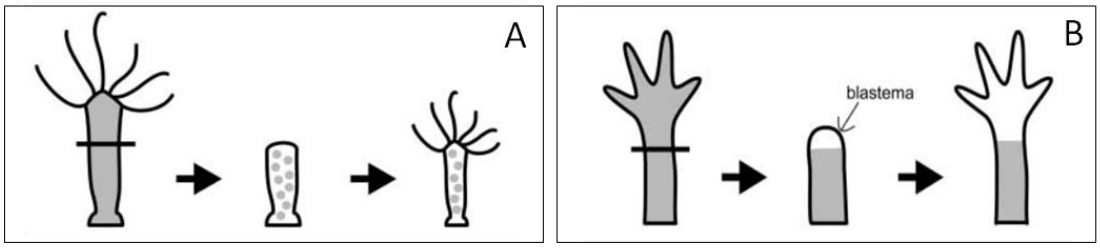

Figure 1.1: Schematic representation of Morphallactic regeneration in *Hydra* (A) and Epimorphic regeneration in Salamnder limb (B). Source: Agata et al. (2007).

An interesting path is taken up by Planarians to regrow the full body from fragments and understanding it is relevant for our understanding of the differences between epimorphosis and morphallaxis. Thomas H. Morgan (1900) called it morphallaxis; however, the presence of a blastema convinced many others that it was actually epimorphic in nature. And yet, as pointed out by Agata et al. (2007), planarian regeneration cannot be unambiguously classified under either of these types.

Research at cell and molecular levels by various groups (Umesono et al., 1997; Agata et al., 1998; Kobayashi et al., 1999; Shibata et al., 1999; Cebrià et al., 2002; Ogawa et al., 2002; Hayashi et al., 2006) has changed the popular view about regeneration in Planaria. Agata et al. (2007) are of the opinion that the current classification of regeneration must be reconsidered to accommodate the events occurring at the cellular and molecular levels. According to their review, regeneration in all animals involves formation of a distal structure shortly after amputation, in a process called distalization, and this distal structure (Wound epidermis or Blastema) interacts with the stump tissue 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). In fact, these authors also suggest that the slow progress in the field of regeneration, as discussed earlier, could well have been due to imperfect understanding and interpretation of some of its underlying principles.

Regardless of the mechanistic differences among them, Hydra, Planaria, Zebrafish and Salamanders almost always find their way into every discussion on regeneration in literature.

MODELS OF REGENERATION STUDIES

Of the standard classical model organisms used over the years for studies on developmental mechanisms, intriguingly, none are capable of significant regeneration. This has resulted in a great lag in the understanding of regenerative processes (Bryant et al., 2002), although the models were perfect for the investigation of embryogenesis. Among the animals that went under the lens for studies on regeneration, the ones that attracted most of the attention were Hydra, Planarians, Zebrafish and Salamanders.

Figure 1.2: Hydra, Zebrafish and Salamanders have been among the most popular models for research in epimorphic regeneration.

Although Zebrafish joined the bandwagon much later than others, it has allowed an appreciable amount of information to be generated, owing to its well established status as an animal model of development. As reviewed by Gemberling et al. (2013), it has had several advantages as a model organism including large clutch size, rapid external development, transparent eggs and a reasonably small genome. The zebrafish has become a frontrunner in vertebrate regeneration research after its strong potential as a model for this area was realised. It is known to able to regenerate fins, heart, CNS structures, jaw, pancreas, liver and kidney (Hata et al., 2007; Pisharath et al., 2007; Anderson et al., 2009; Brignull et al., 2009; Moss et al., 2009; Diep et al., 2011; Anderson et al., 2012; Chitnis et al., 2012; Wang et al., 2012; Shin et al., 2012; Gemberling et al., 2013; Li and Wingert, 2013). It is interesting that after the discovery of fin regeneration in teleost fish, it took over two centuries for the first genetic analysis of the same (Gemberling et al., 2013). It was in 1995 that Johnson and Weston described a screen for mutations in Zebrafish that prevent regeneration of its fin. The model has a number of advantages over other regeneration systems with respect to ease of manipulation at the genetic level. Citing some disadvantages, there has been limited success in generating conditional loss-of-function alleles. Over the years, however, Zinc finger nucleases (ZFN), TALENs and the CRISPR-Cas system have helped in directed mutagenesis.

If one can harness all the existing technologies in Zebrafish to understand regeneration, we will go a long way in elucidating its mechanisms.

The tail-less amphibian *Xenopus* is a very well studied model for the understanding of development. As reviewed by Tseng and Levin (2008) the large size of its embryos has been a factor of convenience in this regard. *Xenopus* larvae – tadpoles – are capable of regeneration and have many advantages as a model system: (i) Embryos develop quickly into tadpoles; (ii) Tails are prominent and easy to ampute; (iii) Tails are transparent and a variety of microscopy techniques can be used; (iv) Embryos are easy to culture in large numbers; (v) Many genetic techniques are available and the genome is very well characterised. Studies in tail regeneration on *Xenopus* tadpoles have indeed generated a thorough understanding of the process in vertebrates.

Among the vertebrates, urodele amphibians are the only tetrapods with the ability to regenerate an array of structures such as limbs, tail and spinal cord throughout their lives (Roy and Lévesque, 2006; Fior, 2014). Salamanders, Newts and Axolotls have been subjects for wide-ranging studies on tissue regeneration, since these are the most evolved vertebrates with such extraordinary regenerative capabilities. The major advantage offered by this group over the ones mentioned earlier is the complexity of the limb structure, which is closer to that of mammals (Alibardi, 2009). Anatomically, fish fins and tadpole tails are fairly simple and in order to understand the dedifferentiation and differentiation of all tissues of the vertebrate limb, the urodeles are a better, if not perfect, example. Much is not discussed about the process of regeneration in Salamanders at this point, since it is similar to that in lizard tails, which will be described later at length.

Regeneration in reptiles

From a regeneration point of view, a gradual decline is seen from lower vertbrates like fish to amphibians and reptiles in the ability to regenerate. This sharply declines in birds and mammals. While lizards have been reported to be able to regenerate multiple tissue types including nerve cells, lens, mandibles and parts of the limb (Simpson, 1961; Bryant, 1970; Bellairs and Bryant, 1985), the most notable example among them is the regenerating tail. Among the reptiles outside Lacertilia, the Crocodilians have been documented for significant tail regeneration and for regeneration of large portions of maxillae (Brazaitis, 1981; Han et al., 2005). Very little is known about regeneration in snakes. Turtles, however, do repair

damages in their shells (Bellairs and Bryant, 1985), although tail regeneration in their case is far from common.

LIZARD AS A MODEL FOR REGENERATION STUDIES

In contrast to Zebrafish and Salamanders, when one considers the higher vertebrates – the amniotes – one finds a sharp decline in regenerative ability. In fact, the only amniotes capable of large scale organ regeneration are some of the lizards. It comes as a surprise why lizards were (and still are) not at the forefront of regeneration research for all these years, given their close proximity to mammals. Lizards are perceived as an 'unpleasant' model to work with and some even argue that they are too far from humans to be used for translatable research (Simpson, 1970; 1983; Simpson and Duffy, 1994). To this, we disagree since in the field of regeneration, although a vast majority of the work is being carried out in the amphibian model, it is the lizards that share more similarities with mammals than do amphibians. As observed by Alibardi (2009) the histological features between reptiles and mammals are similar – definitely more similar than those between the amphibians and mammals. In the evolutionary context as well, lizards are closer to mammals than are amphibians. Reptilia has been called as the 'sister group of mammals' by the reptilian genomics working group. They rightly argue, based on whole mitochondrial genomes, nuclear DNA and fossil data, that this phylum shares a very close phylogenetic relation with us. It is to be pondered upon that although a number of reptiles serve as important models in various branches of biological research, there has been no effort (until recently) to generate information on their genomics. Up until very recently, reptiles were the only major lineage of vertebrates for which there was no genome sequence. After the sequencing project was taken up for the genome of the green anole lizard *Anolis carolinensis* in the last decade, there has been some interest in analysing the genes responsible for tail regeneration in the animal. The recent paper by Hutchins et al. in PLoS One (2014) has garnered quite some attention about the use of a lizard model to study appendage regeneration. Geckos, however, are not new subjects in the field of regenerative biology. Our department at The M. S. University of Baroda has used the northern house gecko *Hemidactylus flaviviridis* since at least five decades. The department has carried out extensive research on the histology, metabolism, biochemistry, nervous physiology, endocrinology and molecular biology of regeneration in lizard tail (Shah and Hiradhar, 1974; Kumar and Pilo, 1994; Pilo and Suresh, 1994; Pilo and Kumar, 1995; Sharma and Suresh, 2008; Suresh et al., 2010; Pillai et al., 2013; Buch et al., 2017). Other important contributions in the field of lizard tail regeneration have been made by Lorenzo

Alibardi with various publications on the anatomical, histological, ultrastructural and molecular aspects of the regenerating tail (Alibardi, 2009; Alibardi, 2014). McLean and Vickaryous (2011) have dissected and described the entire process of tail regeneration in the leopard gecko *Eublepharis macularius* in a recent publication. Not many other labs in the world pursue regeneration in lizards as a central focus and therefore, progress in this area has been agonisingly slow, as already stated. Nevertheless, encouraging the use of this model in this area will surely yield interesting results that can be better extrapolated to the human cause. For this, one needs to have an in depth understanding of the mechanism, starting from how it evolved.

Some evidence points towards a concurrent evolution of regeneration with the phenomenon of autotomy. This theory gains credence from the observation that tail regeneration is either absent or rare in species where autotomy does not take place (Werner, 1968; Vitt, 1983; Bellairs and Bryant, 1985; Fitch, 2003; Maginnis, 2006).

Autotomy

Autotomy is the voluntary release of a body structure almost always with the intent of escaping predation or other similar threat. While it is indeed a voluntary action, it is often the result of a reflex response. The phenomenon is most interesting in that it is all about a fine balance between the pros and cons of the process and the outcome. Every adaptation for and implication of autotomy has both – advantages and disadvantages – and is successful in nature based on how these weigh against each other. This trade-off, usually causing a heavy physiological drawback, is aimed at a much bigger cause – survival. The success of autotomy as a phenomenon in the animal kingdom can be testified by the fact that it is exhibited by such diverse groups as crutaceans through mammals, including echinoderms, amphibians and reptiles (Juanes and Smith, 1995; Bernard and Agosta, 2005).

A number of lizards use autotomy of the tail as a strategy to escape predation. As analysed by McConnachie and Whiting in 2003, lizards from 13 of the 20 families possessed this ability. 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. The loss of a tail does have its disadvantages, but these are compensated for by its subsequent regeneration. For instance, the tail is required for

locomotion, social and sexual interactions and most importantly, for energy storage. 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 1.3: A plane of autotomy lies between each segment of the lizard tail and corresponds to the median plane of each vertebra. The plane is visible externally at the junction of the original and the regenerated tail, marked by an arrow (image on left). The vertebral column does not regenerate and instead a hollow rod of cartilage grows out from near the fracture plane, which can be seen in an X-Ray radiograph (image on right).

A voluntary release of the tail is understandably not brought about successfully without certain anatomical and physiological adaptations. Some main features of the autotomy-capable tail include the presence of fracture planes – pre-determined planes of weakness along the centre of each vertebra. Bellairs and Bryant (1985) documented that most lizards have fracture planes in all caudal vertebrae except few closest to the cloaca (called the non-autotomous pygeal series). This, however, is not the case in *Hemidactylus flaviviridis*, which has autotomous vertebrae right up to the proximal-most caudal segment. Moreover, the muscle bundles in the tail are short allowing breakage. A series of muscle contractions in the region of the fracture plane allow for release of the tail from that site. Also, since the blood loss could be lethal, the circulatory system is adapted to minimise blood-loss after tail release through the presence of arterial sphincters and valves (Arnold, 1984). Blood vessels break after these valves and almost no bleeding is seen post autotomy. Apart from the potentially

lethal outcome of blood-loss, a non-lethal but serious implication is the loss of large energy reserves stored within the tail. To overcome this, some lizards, such as the metallic skink *Niveoscincus metallicus* stores most of the fat in the proximal third of the tail, rather than uniformly distributing it. This decreases the likelihood of loss. This observation by Chapple and Swain (2002) is concomitant with our observations in the *H. flaviviridis* (typically for lizards which weigh over 12 g). There are some disadvantages, however, which cannot be overcome. Tailless lizards show reduced reproductive success: small litter size or egg size and mass for females (Dial and Fitzpatrick, 1981; Chapple et al., 2002) and lowering of social status for males (Fox and Rostker, 1982). Whilst their tails regenerate, lizards maintain a 'low profile' since they are most vulnerable during this time. This duration could be prolonged, depending on the species (and other factors like temperature) since regeneration, of course, is a complicated process and takes its time. The process of tail regeneration is discussed in detail below.

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 (reviewed by Bryant et al., 2002). As categorised by Carlson et al. (1998), appendage regeneration in vertebrates proceeds via three major stages: Wound Healing, Blastema formation and Growth & 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 to counter the damage 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 intact epidermis (reviewed by Bryant et al., 2002 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). 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., 2002). During wound healing, an array of proteins is secreted locally. Matrix metalloproteinases (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 pool, called the blastema, is characteristic of epimorphic regeneration. Cells for the blastema 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). 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., 2002). 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 the 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.

Epimorphosis in Lizard

Observations in our lab suggest that tail regeneration in the lizard *H. flaviviridis* follows the above-stated steps without any deviation (Figure 1.4). The wound epidermis or AEC forms from migrating epithelial cells. AEC formation is completed on the 4^{th} or 5^{th} day post amputation (dpa) at 37°C. Tissues from the underlying mesenchyme simultaneously reorganise and accumulate under the epithelial cap. After AEC formation, a blastema is seen to bulge immediately and a prominent conical blastema can be observed at 6^{th} or 7^{th} dpa. The late blastema, from the 9^{th} dpa enters the growth and differentiation stage with the distal end consisting of fast proliferating blastemal cells and the proximal end gradually differentiating into the constituent tissues of the tail.

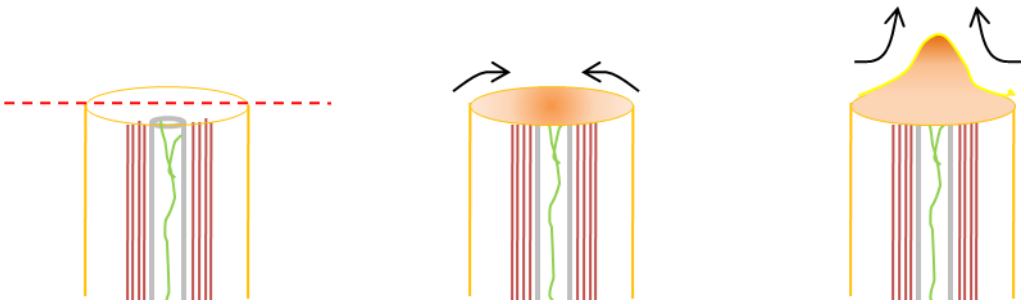

Figure 1.4: Schematic diagram of the stages of regeneration in lizard tail. Red dashed line represents the plane of amputation (left). Re-epithelialisation occurs as a result of migration of epithelial cells from the wound margins and this results in formation of wound epithelium, followed by the AEC (centre). Shortly after AEC formation, a bulged blastema is seen due to rapidly accumulating progenitor cells underneath the AEC (right).

There is, however, one major difference in the case of lizard tail regeneration from that in urodele amphibians (Alibardi, 2009). The spinal cord and the vertebral column do not regenerate. The innervation of the new tail is achieved by growing axonal processes from the cut end of the spinal cord. The vertebral column is replaced by an unsegmented rod of cartilage (Figure 3), which does show ossification early on (personal observation), but does not form vertebrae. This is the reason the regenerated lizard tail is not considered a perfect replica of the original.

A number of molecular signals work in a co-ordinated manner for the timely achievement of each of the above-mentioned stages of regeneration and also for smooth transitioning from one to another. A fairly large amount of research has gone into elucidating the function and regulation of several growth factors in this regard. However, the part played by inflammatory cytokines remains a grey area in epimorphic regeneration. Keeping in line with the key

immune regulator on which the present thesis is based, a brief review of the prostanoids is taken below.

PROSTAGLANDINS

Prostanoids are a group of Eicosanoids (derivatives of eicosanoic acid) involved in diverse biological reactions including inflammation, cancer, allergy, fever, blood pressure, etc. They were discovered after von Euler (1936) showed that injecting semen into animals lowered their blood pressure. A number of other functions were attributed following further research over the years.

Eicosanoic acid, better known as Arachidonic acid, is a 20-carbon fatty acid with four double bonds, as seen in figure 1.5. This fatty acid, along with other similar polyunsaturated fatty acids (PUFAs), is a precursor to various biologically important molecules grouped as the Prostaglandins and the Thromboxanes. The prostaglandins (PGs) contain a cyclopentane ring. These are further grouped into Prostglandin A through I, on the basis of modifications on the cyclopentane ring. Among these, Prostaglandins D to I are naturally occurring. Within each group of compounds (A to I), each member is named with a number that represents the number of double bonds present in the side-chains (reviewed by Bos et al., 2004).

The series 2 compounds (with two unsaturated sites) are all synthesised from Arachidonic acid through a series of enzyme catalysed reactions. Since arachidonic acid is the most abundant of the prostanoid precursors, the series 2 prostanoids are most commonly found (Bos et al., 2004). And among the series 2 prostanoids, the one relevant to the present discussion, and also the most widely studied molecule, is Prostaglandin E_2 (PGE_2).

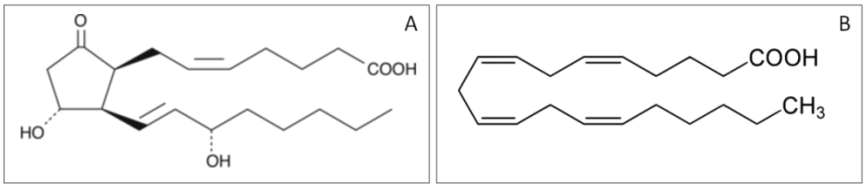

Figure 1.5: PGE_2 is a series 2 prostanoid as seen by the number of double bonds in its side chains (A). Series 2 prostanoids are biologically synthesised from Arachidonic acid (B).

Prostaglandin E_2

PGE_2 is a product of Arachidonic acid, which is released from membrane phospholipids in a reaction catalysed by Phospholipase A_2 (PLA_2). There are 15 groups of PLA_2 (according to Schaloske and Dennis, 2006) classified into four categories: (i) Secreted $sPLA_2$; (ii) Cytosolic

PLA_2; (iii) Calcium dependent $iPLA_2$; (iv) Platelet activating factor (PAF) acetyl hydrolase/oxidised lipid lipoprotein (Lp) associated PLA_2. PLA_2 activation results from tissue damage and signalling from extracellular factors like TNF-α and EGF. It hydrolyses fatty acids from the Sn-2 position of membrane phospholipids (Burke and Dennis, 2009). The Sn-2 position of membrane phospholipids often contains PUFAs and thus the activation of PLA_2 results in release of Arachidonic acid, an n-6 PUFA. This release is a rate-limiting step in prostanoid biosynthesis.

The arachidonic acid thus released is first enzymatically converted to Prostaglandin G_2 (Hamberg and Samuelson, 1973) by cyclisation and oxygenation by a Cyclooxygenase. The same enzyme further reduces a hydroperoxide (-OOH) group to hydroxyl group (-OH) forming PGH_2. From PGH_2, various enzymes, by means of isomerisation or redox reactions, produce numerous prostaglandins. PGE synthase catalyses the final step in the formation of PGE_2 by isomerisation of PGH_2 (Reviewed by Bos et al., 2004 and Simmons et al., 2004).

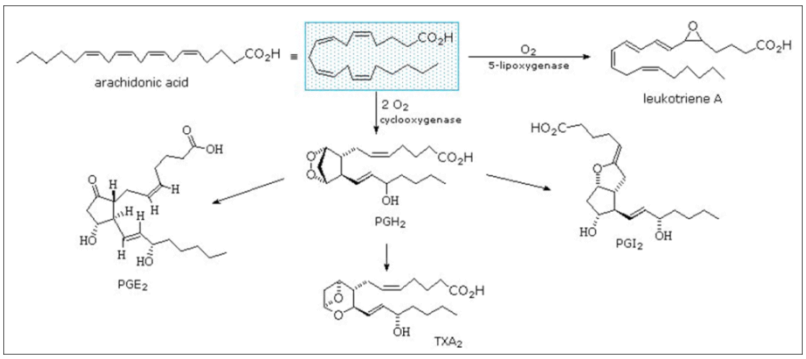

Figure 1.6: The arachidonic acid pathway

Cyclooxygenase

The cyclooxygenation reactions involved in synthesis of the prostanoids are catalysed by the Cyclooxygenase (COX) enzymes. COX, also called Prostaglandin H synthase or Prostaglandin endoperoxide synthase, is the key enzyme in the oxidative conversion of Arachidonic acid to PGH_2 (Smith and Lands, 1972; Hamberg et al., 1974). It was observed that prostaglandin synthesis and release in some situations, such as activated platelets, occurred almost instantaneously after stimulation. In some other cases, such as in mitogen-activated fibroblasts, prostaglandin synthesis took hours to occur. These observations eventually led to the discovery of multiple isoforms of COX (reviewed by Simmons et al., 2004).

COX-1 is constitutively expressed (O' Neil et al., 1994) and is essential for maintenance of normal physiological functions. COX-2, on the other hand, is an inducible isoform expressed in response to stimuli such as cytokines and endotoxin. The inflammatory effects of prostaglandins are mainly associated with the induction of this isoform (Maier et al., 1990; Lee et al., 1992; Xie et al., 1992). Sequences of COX-1 and COX-2 (from chicken and mammals) reveal approximately 60% amino acid identity between the isoforms. The structures of both the isoform typically represent their localisation in the lumen of nuclear envelope and endoplasmic reticulum. Disulphide bonds in their structures are also in agreement with their location (Simmons et al., 1991).

The COX enzymes have a central role in a number of human diseases associated with inflammation. They have thus been targets for many pharmacological drugs. Non-steroidal anti-inflammatory drugs (NSAIDs) are used in the treatment of conditions like Osteoarthritis and Rheumatoid arthritis and other inflammatory diseases. Of course, these provide symptomatic relief rather than cure, by alleviating inflammation and pain. The NSAIDS, however, have damaging effects on the gastric mucosa and cause ulceration. Nimesulide, Etodolac and Meloxicam were developed in the 1980s as NSAIDs with less side-effects on the stomach. Later, after the discovery of COX-2 in 1991 (Kujubu et al., 1991; Xie et al., 1991) inhibitors were designed with more specificity for the COX-2 isoform. Selective COX-2 inhibitors have been popular anti-inflammatory agents since then and their rampant use underlines the important place of COX-2 in human pathology.

Of all the COX metabolites, PGE_2 has been most widely studied for its cellular and molecular effects (reviewed by Simmons et al., 2004). It is best known as a pro-inflammatory compound. It regulates the production of various cytokines and immune molecules. However, the downstream effects of PGE_2 are multiple and sometimes seem to be functionally opposing. This multiplicity of effects is mediated by a group of receptors expressed in different cell types. PGE_2 is found in the synovial fluid of the knee joints of arthritic patients (Higgs et al., 1974; Brodie et al., 1980; Bombardieri et al., 1981). It was also found to be the cause of inflammation and pain in the rat model of Carrageenan-induced paw edema (Mnich et al., 1995; Portanova et al., 1996). PGE_2 also mediates the pain induced by pain-mediating molecules such as Bradykinin and Histamine (Ferreira, 1972). It is known that fever is caused by PGE_2 released from endothelial cells lining the blood vessels of the hypothalamus, which is responsible for controlling body temperature (Cao et al., 1998). It is interesting that PGE_2

has significant immuno-suppressant consequences as well. It has been suggested that it is the PGE_2 produced by tumor cells that is responsible for the repression of the immune system – a common feature of cancer systems. Plescia et al. (1975) and Bennet et al. (1977) have noted that large amounts of prostaglandins are produced by some tumor cells, resulting in an immunodeficient environment.

OBJECTIVES OF THIS STUDY

The current project was aimed at elucidating the mechanism through which Cyclooxygenase-2-mediated PGE_2 assists in bringing about successful regeneration in vertebrates. This was achieved using three specific objectives:

1. The inflammatory mediators during early epimorphosis with special reference to COX-2.
2. Effect of COX-2 inhibition on wound healing in regenerating system.
3. Effect of COX-2 inhibition on blastema formation in regeneration.

REVIEW OF LITERATURE

Lizards have been identified as noteworthy for their unique ability among the amniotes to regenerate an entire tail. However few studies have addressed the process from a mechanistic point of view. Research at the department of Zoology at our university has involved varied aspects of lizard tail regeneration since the 1960s, as mentioned earlier in this chapter. Very few labs elsewhere have dealt with regeneration in this model organism. The labs of Alibardi and Vickaryous have pursued regeneration in lizards as a line of research.

Alibardi has discretely described the morphological aspects of tail regeneration in a lizard and has backed these up with a series of observations on the ultrastructure as well (Alibardi, 2009). He has described, with detailed histological evidence, the various tissues and their differentiation during the course of regeneration. A more recent publication (Alibardi, 2014) reviews his own work on the biochemistry and metabolism in the regenerating tail, building up on the extensive research carried out through the 1970s and 80s at our university in Vadodara.

Hutchins et al. (2014) attempted to identify the major genes involved in tail regeneration in the green anole *Anolis carolinensis*. With a transcriptome level analysis, they have been able to draw a comparison between the processes in amphibians and reptiles.

As far as the role of inflammation in regeneration is concerned, little data is available for the higher vertebrates. It is known, from research carried out by Mescher's lab on an Anuran model, that while an inflammatory response is critical to the success of epimorphic regeneration, its timely resolution is also of prime essence. Their work showed that prolonged inflammation, as also premature resolution of inflammation, is detrimental to the process of regeneration (King et al., 2012; Mescher et al., 2013). Petrie et al. (2014) have presented a definitive link between immune cells and the formation of wound epithelium and further regeneration of Zebrafish fin. The regulation of the inflammatory environment was found to be very important for proper fin regeneration.

Literature, however, has almost no information on the relevance of the Cyclooxygenase pathway for epimorphosis. In this regard, a pertinent piece of research is one by Appukutan et al. (1993). They monitored the metabolism of PGE_2 during blastema formation and also described its role in differentiation of tissues from blastemal cells. Work described in the present thesis is rooted in a more recent piece of research by Sharma and Suresh (2008) which confirms that Cyclooxygenase-2 mediated PGE_2 is vital for wound healing and blastema formation in tail regeneration. What remains to be understood is how PGE_2 influences these processes and what the outcome is of PGE_2 inhibition with respect to the major signalling pathways involved therein.

ORIGIN OF THE PROBLEM

With the turn of the century, as investigations in our department moved from the anatomical and physiological aspects of regeneration to its biochemical and molecular aspects, the role of a number of enzymes and the induction and function of a number of essential growth factors came to light. Among the most recently studied growth factors in our lab are the Epidermal Growth Factor (EGF) and Fibroblast Growth Factors (FGF). Exogenous administration of both these factors separately was found to enhance the process of regeneration in lizards (Pillai et al., 2013; Yadav et al., 2014). EGF added externally resulted in an increased rate of cell proliferation, as seen in the BrdU experiments carried out in vivo (Yadav et al., 2014). Moreover, the effect of FGF signalling was confirmed using SU5402, a pharmacological inhibitor of FGFR1, in a series of experiments on both lizard and teleost fish (in caudal fin regeneration). SU5402 administration caused a retardation of the regenerative processes in both the model organisms. It was detrimental to the achievement of both the major milestones of epimorphic regeneration, *viz.*, Wound Healing and Blastema formation (Pillai et al., 2013).

It was in 2004, that investigations on the involvement of Prostaglandin E_2 in lizard tail regeneration commenced in our lab. The work was initiated on the basis of the well known role of PGE_2 as a major mediator of inflammation, and that the regulation of inflammation must be crucial to the success of regenerative healing. There were, up to then, almost no reports of Cyclooxygenase-2 mediated PGE_2 production being important for epimorphic regeneration.

In the work of Priyanka Sharma (2008), the specific effects of COX-2 mediated PGE_2 were revealed by using inhibitors specific for the isoform. After selection of an appropriate inhibitor with high specificity for COX-2 and after a dose-range study, Etoricoxib was used. Local injections in the tail prior to induction of autotomy (hereafter referred to as 'amputation') altered the course of regeneration in lizards. The drug delayed the process of wound healing and also deferred blastema formation significantly. Etoricoxib administered after amputation also showed similar results and a high dose of 50 mg/kg body weight per day caused a growth arrest until cessation of drug administration.

To look into the interactions of or immediate downstream effects of PGE_2, various parameters in the regenerating tail were assayed. Etoricoxib resulted in a decrease in the activities of the major antioxidative enzymes. Superoxide dismutase (SOD), Catalase, Glutathione peroxidase, Lipid peroxidation estimations reflected a heightened oxidative environment locally in the tail in response to COX-2 inhibition across all stages of regeneration (Sharma, 2008).

SDS-PAGE was used to check for any changes in the protein expression patterns in the treatment groups as compared to control. COX-2 inhibition had caused a change in the expression of a number of peptides, as revealed by their relative intensities on the gels. Further, the Matrix metalloproteinases, which are vital in the formation of the wound epidermis and in the outgrowth during the later stages, were also found altered in their activities, as seen on the gelatin zymograms. DNA synthesis was found reduced and cellular synthetic activities and cell proliferation had slowed down, as seen in BrdU incorporation experiments (Suresh et al., 2009).

In the later stages, muscle specific molecules Myosin and Desmin and the angiogenesis marker VEGF were reduced in expression, reflecting an alteration in formation of the integral tissues, which characterises regeneration (Suresh et al., 2009).

RATIONALE OF THE STUDY

Whilst the above investigation on lizard tail regeneration confirmed the involvement of Cyclooxygenase-2, it was strongly believed that a further insight into the mechanism therein would immensely benefit the field of regeneration.

The choice of animal model

Our lab focuses on *H. flaviviridis* model for a number of reasons, the primary one being its place in evolution. Reptiles are closely placed with mammals and therefore, any information generated from lizards can be handy for replicating the process in humans. After all, regeneration research is taken up with an ulterior motive of developing techniques to induce large scale tissue regeneration in humans. Also, among the amniotes – a class of organisms to which we belong – the lacertids are the only group that possess the ability to restore appendages. Thus, if one is to study the process in amniotes, one has to look to this group of animals. All other amniotes are incapable of epimorphic regeneration in adult life (with exceptions such as deer antlers, which do not share the tissue complexity and are therefore not suitable models). More justification on the choice of the lizard model is provided in the section 'Lizard as a Model for Regeneration Studies' above.

Moreover, parallel work in the lab deals with the role of the COX pathway in teleost fish *Poecilia latipinna*. While throwing light on the contribution of PGE_2 in regeneration in both the animal groups, the data from these two studies will also enable us to draw a comparison between mechanisms in lower and higher vertebrates.

PGE_2 as a target

As already mentioned, much of the research on epimorphosis has dealt with the interplay of growth factors and developmentally important molecules. However, immune regulators like Interleukins, Proastanoids, etc. have received very little attention. It is widely accepted that regulation of the immune response is crucial in regenerative success and thus explicating the position of the COX-2 pathway therein seems necessary.

The choice of stages

This thesis incorporates results from experiments carried out on the two early stages of regeneration, *viz.*, Wound healing and Blastema formation. This is because the redevelopment phase, i.e. the growth and differentiation stage is very similar to embryonic development. Studies on this phase have concluded that regeneration and development are

very similar (Bryant and Gardiner, 1992). It is the very early stages that are in fact very different and need to be paid attention to. As pointed out by Han et al. (2005), the process leading to healing in regenerating animals is different from that in non-regenerating animals right from the time-point of injury. They suggest that regeneration studies must focus on the earliest events therein, if one hopes to alter the course of mammalian wound healing towards a regenerative type.

Why study regeneration?

What could be called a fantasy in the 18th century, has suddenly started to seem possible in the 20th century. With enormous advances in technology at hand, scientists have come to believe that regeneration, at least to some extent, can artificially be induced in humans. This is not limited to amputation injuries, and can be extended to diseases involving tissue degeneration such as Alzheimer's and Parkinson's. It is a strong personal view that as opposed to injection of stem cells for large scale tissue renewal, we must look to activate endogenous stem cell niches to achieve tissue restoration. For this, basic research on the mechanism of epimorphosis becomes inevitable. It is a vital approach which must continue to be pursued parallel to advances in tissue engineering. Moreover, regenerating systems hold immense potential for use in cancer biology. If a thorough understanding of the regulation of proliferation during regeneration can be attained, it can prove useful for application to cancer cells. One may possibly be able to lead cancer cells to the path of differentiation and thereby control them.