Regeneration is the ability of an adult organism to remake the damaged or the completely lost body parts or the organs or the tissues. The process of recovery is further called restitutive regeneration when the lost part is reformed and capable of performing the complete or partial physiological activity performed by the original, lost body part (Alibardi, 2010). This involves recapitulating part of the embryonic development. Some tissues, such as blood and epithelia, undergo continual turnover and thus must replace themselves continually, a process called maintenance or homeostatic regeneration. These tissues as well as a number of others regenerate on a larger scale when damaged and hence, the process is christened injury-induced regeneration (Stocum, 2006). The relationship between regeneration, life and death has been concisely explained by one of the great masters of regenerative biology, R. J. Goss as “If there were no regeneration there could be no life. If everything regenerated there would be no death. All organisms exist between these two extremes. Other things being equal, they tend toward the latter end of the spectrum, never quite achieving immortality because this would be incompatible with reproduction”. In other words, individuals in a species are in a constant battle that pits their ability to locally reverse the second law of thermodynamics (regenerate) against inexorable entropic processes, a battle that each one ultimately lose as individuals, but win as a species through reproduction (Goss, 1969).

Regeneration has always been a topic of interest in the history of science and medicine. Primitive people were likely aware of the ability of certain food animals, such as male deer and elk, to shed and regenerate antlers, and crayfish or lobsters to regenerate limbs. They were undoubtedly cognizant of the fact that hair and nails grow continually. The regeneration of organs and appendages is a theme found in the ancient Greek myths of the Hydra’s ability to regenerate its many heads, and of chained Prometheus condemned to watch his own liver regenerate every time it was devoured by an eagle (Dinsmore, 1998). Regeneration became a focus of systematic scientific investigation in the 18th century. Abraham Trembley showed that hydra can regrow into complete animals after being cut into several pieces (Dinsmore, 1991), while Reaumer and Spallanzani reported observations on the regeneration of limbs in crustaceans and newts respectively (Dinsmore, 1991; Skinner and Cook, 1991). In the early 20th century, T.H. Morgan systematically studied the ability of planaria to regenerate even after being cut into over 100 sections (Morgan, 1901). Subsequent studies have continued to focus interest on animals with high regenerative abilities (Birnbaum and Sanchez-Alvarado, 2008).
Regeneration is widespread in the animal kingdom. Ungulate antlers, turtle shells, crocodilian jaws, bat wings, and snail penises have all been shown to regenerate (Bellairs and Bryant, 1985; Goss, 1987; Dytham et al., 1996). In a few taxa, regeneration can even serve as a means of asexual reproduction eg. earthworms, poriferans and asteroids. Among vertebrates, fish can regenerate their fins and heart upon amputation. The amphibians have an extensive power of regeneration, wherein urodeles can regenerate their limbs, tails and jaws and snout while anurans can regenerate their hindlimbs and tails in larval life. Among reptiles, only the tail of lizards and shell of testudines show extensive regenerative abilities. Mammals which are the highest form of life, lack any such capability of regenerating the organs once lost, but can regenerate some part of tissues like liver, muscle, bone, blood and epithelia (Uchida et al., 2000). However, the most common forms of regeneration – and those that are best characterized developmentally – involve appendages, such as regeneration of legs and tails in amphibians and lizards (Maginnis, 2006). The capacity of certain animals to regrow a lost leg or tail was exploited as a powerful tool by biologists to study the fundamental aspects of development, such as wound healing, blastema formation, and cell differentiation / growth. As a result, we now understand many of the mechanistic details of the regeneration process at the genetic, cellular, tissue, organ, and organismic level.

The canonical response to an injury by an organism is to initiate the process of wound healing. The primary goal of wound healing is to re-establish homeostasis and restore tissue architecture. For this, nature has provided us with a mechanism for injury-induced repair, the fibrosis, which is the result of an inflammatory response to injury that produces a fibroblastic granulation tissue that is then remodelled into a virtually acellular collagenous scar. Once formed, this scar not only alters tissue function and physiology, but also appears to inhibit regeneration (Ferguson and O’Kane, 2004). Hence, fibrosis maintains the overall integrity of the tissue or organ, but at the expense of reducing its functional capacity. Mammalian tissues that do not regenerate spontaneously are repaired by fibrosis. Prominent examples of tissues that undergo repair by scar tissue when injured are the dermis of the skin, meniscus and articular cartilage, the spinal cord and most regions of the brain, the neural retina and lens of the eye, cardiac muscle, lung, and kidney glomerulus. It is not that these tissues have no ability to regenerate. Many, if not all, initiate a regenerative response to injury, but the response is overwhelmed by a competing fibrotic response. In contrast animals with regenerative capacity such as urodeles and lizards undergo wound healing without the formation of a scar tissue. As a result, it has been proposed that scar-free wound healing is a requirement for reparative regeneration (Ferguson and O’Kane, 2004; Metcalfe and Ferguson, 2007; Gurtner et al., 2008; Yokoyama, 2008; Occleston et al., 2010).

Although less common, various examples of mammalian scar-free wound healing have been documented viz. liver regeneration (Fausto and Campbell, 2003), infant finger tips (Han et
al., 2005), scar-free wound healing of punched ear holes in rabbits, bats, cats (Metcalfe et al., 2006) and the genetic variant MRL mouse, and in utero incisional wounds in mammal embryos (Whitby and Ferguson, 1991; Ferguson and O’Kane, 2004). While the molecular details of scar-free healing remain poorly understood, it is widely suggested that minimal/attenuated immunological response of urodeles (when compared to mammals) plays a crucial role in inhibiting scar formation (Cowin et al., 1998; Harty et al., 2003; Godwin and Brockes, 2006; Occleston et al., 2010).

**TYPES OF REGENERATION**

As first noted by Morgan, regeneration in the metazoans can be classified into two groups according to the following criteria: 1. Regeneration which occurs in the absence of active cell proliferation, and 2. Regeneration which requires cell proliferation. The first is referred to as **Morphallaxis**, which involves the re-creation of missing body parts solely by the remodelling of pre-existing cells. An example of morphallactic regeneration is provided by Hydra which utilizes stem cells found in the gastric region to regenerate itself or its lost structures (Bosch, 1998). The second mode of regeneration was originally termed **Epimorphosis** by Morgan (Morgan, 1901). Currently, epimorphic regeneration is subdivided into two broad categories - non-blastemal and blastemal based regeneration. Non-blastemal regeneration occurs as a result of: a) transdifferentiation of the remaining tissue into the missing structure e.g. Lens regeneration in urodele amphibians (Reyer, 1954) b) limited dedifferentiation and proliferation of the surviving cells in the organ after injury or amputation e.g. Liver (Michalopoulos and DeFrances, 1997); and c) by the proliferation and differentiation of stem cells already present in the damaged tissue e.g. Mammalian muscle regeneration (Uchida et al., 2000).

Blastemal based regeneration, on the other hand, involves the formation of a specialized structure known as regeneration blastema. This structure, similar in form and organization to the early embryonic limb buds produced during vertebrate embryogenesis, is a population of mesenchymal progenitor cells that is necessary for proliferation and patterning of the regenerating part. The missing parts are regenerated by the eventual differentiation of the blastema. Blastemas have been described in planarians (Egger et al., 2007), molluscs (Flores et al., 1992), echinoderms (Thorndyke and Carnevali, 2001), crustaceans (Hopkins, 1993), teleost fish (Poss et al., 2003), urodele amphibians (Nye et al., 2003), larval anuran amphibians (Yokoyama, 2008), lizards (Clause and Capaldi, 2006) and in some mammalians (Han et al., 2008). Great debate has ensued over the origin of these blastemal cells and to characterize their cellular and molecular nature has been a major goal of regeneration biologists (Whited and Tabin, 2009). The planarian blastema is formed from pre-existing stem cells called neoblasts (Baguna and Slack, 1981), whereas blastema in the regenerating axolotl tail arises by the reprogramming and de-differentiation of differentiated cells.
(Namenwirth, 1974; Kintner and Brockes, 1984; Casimir et al., 1988; Lo et al., 1993; Echeverri et al., 2001; Brockes and Kumar, 2002; Echeverri and Tanaka, 2002). However, activation of resident muscle stem cells has also been reported in regenerating salamander limbs (Morrison et al., 2006). Thus, it is likely that dedifferentiation and stem cell activation both contribute to formation of the blastema. Moreover, a lineage study of regenerating salamander limbs using transgenesis of GFP (Green Fluorescent Protein) labelled cells revealed very little transdifferentiation between cell types, suggesting that the blastema is not a homogenous population of molecularly identical cells but is instead heterogenous from its inception, a conclusion that challenges the notion that complete dedifferentiation is a major force behind blastema creation (Kragl et al., 2009).

Other definitions of regeneration types have been given by Stoick-Cooper et al. (2007) as follows:

**Compensatory growth**: here it is not the damaged part of an organ that is restored, but uninjured parts of the organ compensate for the loss by growth (e.g., after removal of two lobes of the liver, the third lobe grows until the original mass of the liver is restored).

**Tissue regeneration**: repair of local, limited damage to an organ predominantly via restoration of only one cell type (e.g., skeletal muscle).

These regenerative processes include phenomena of very different complexities: compensatory growth of the liver after partial hepatectomy and repair of local damage to muscle are both certainly much less complicated processes than regrowth of a limb containing many different cell types that are organized into tissues and patterned along the proximal–distal, dorsal–ventral, and anterior–posterior axes. Nevertheless, all regenerative processes need to be tightly regulated and involve communication between different cell types.

**EPIMORPHIC REGENERATION: STAGES AND INFLUENCING FACTORS**

Epimorphic regeneration is a post traumatic morphogenetic event characterized by the aggregation of proliferating cells at the wound site (Carlson, 2007). It involves the closure and re-epithelialization of the wound and the recruitment of mesenchymal cells to form a blastema which is a mass of non-differentiated, proliferating cells, located beneath the wound epithelium. This epithelium forms an apical cap, which is thought to direct the regenerative process (Mescher, 1976; Tassava and Garling, 1979; Lheureux and Carey, 1988; Ferretti and Geraudie, 1995; Neufeld and Day, 1996). Continuous proliferation in the blastema causes structure outgrowth by providing new cells, which will differentiate into all the different mesenchymal cell types needed to rebuild the lost body part. The mitotic rate of the blastema slows down as the structure grows, and it ceases completely when the new structure reaches
the original size (Maden, 1976; Wallace and Maden, 1976; Smith and Crawley, 1977; Tomlinson et al., 1982; Santamaria et al., 1996). However, such a complex process requires precise coordination of cell proliferation, cell differentiation, morphogenesis, and pattern formation. Cell proliferation is likely to be controlled by a series of specific mitogenic and anti-mitogenic signals, which drive multiple pathways within the cells.

Experimental studies in several regeneration models, particularly urodele amphibians, have led to identify the major factors regulating epimorphosis during its different stages. A brief overview of these factors is as follows:

**Wound healing**

Following injury or amputation, surface of the wound is covered by epidermal cells migrating from the edge of the amputation surface forming the wound epidermis (WE) (Call and Tsonis, 2005). It is not known what immediate signals induce cells to migrate to cover the wound, but it is known that the formation of the WE is required for regeneration to occur (Thornton, 1957). Matrix metalloproteinases (MMPs) are up-regulated very early after amputation and are required for regeneration, and it is postulated that they play a role in matrix degradation, contributing to formation of the WE (Call and Tsonis, 2005; Vinarsky et al., 2005). The WE becomes a specialized structure - the apical epithelial cap (AEC), which is distinct morphologically and in gene expression from the normal epithelium (Call and Tsonis, 2005; Han et al., 2005). This structure is thought to be similar to the apical ectodermal ridge (AER) that is present in the developing limb bud, which directs and patterns limb outgrowth in amniotes (Summerbell, 1974; Saunder et al., 1976; Saunders, 1998), but there is some debate about how similar these structures actually are, since after amputation of a developing limb with an AER (like the chick limb bud), the AER does not regenerate, and neither does the developing limb (Tschumi, 1957; Hayamizu et al., 1994). Recent evidence shows that Wnt/β-catenin signalling is required for structural maturation of the WE in axolotls, frogs and fish but not for the earlier phase of epidermal migration after amputation (Poss et al., 2000; Kawakami et al., 2006).

**Blastema formation**

As described earlier, blastema formation is the result of cell dedifferentiation and/or resident stem cell activation. Tanaka et al. (1999) have shown that the blood clotting proteinase thrombin may act as an extracellular signal that induces muscle dedifferentiation, as it can indirectly induce S-phase re-entry in cultured newt myotubes. Intracellularly, phosphorylation of the retinoblastoma (Rb) protein and expression of the homeobox protein msx1, a transcriptional repressor that is expressed in many regenerating systems, is also known to be required for myotube cell cycle re-entry in vitro (Tanaka et al., 1997; Kumar et al., 2004).

*Introduction*
Many studies describe the role of the dermis in contributing to the formation of the blastema; it is distinct in this way from the epidermis, which is known to only contribute to the formation of the WE (Riddiford, 1960; Hay and Fischman, 1961; Endo et al., 2004). Cells of the dermis can give rise to multiple cell types in the regenerating limb, including cartilage and connective tissue (Dunis and Namenwirth, 1977). Further, Wnt7a is known to induce dedifferentiation of mammalian chondrocytes *in vitro* by stimulating β-catenin mediated transcription (Hwang et al., 2004a).

Signals from the WE are thought to induce formation of the regeneration blastema. Among these, Fibroblast growth factor (FGF) signalling is an important requirement of regenerative ability, particularly, expression of FGF2, FGF4, FGF8 and FGF10 (Taylor et al., 1994; Kostokopoulou et al., 1996; Yokoyama et al., 2001; Christensen et al., 2002). Beck et al. (2006) showed that BMP signalling is required for blastema formation, msx1 and FGF8 expression, and proliferation of cells in the epidermis as well as the blastema during *Xenopus* regeneration. In fact activating msx1 in transgenic frogs amputated during the refractory period stimulates normal regeneration suggesting that msx1 can substitute for BMP signalling in tail regeneration and is likely an important regulator of the mechanism by which BMP signalling stimulates regeneration (Beck et al., 2003). Notch signalling inhibition completely abolishes tail regeneration suggesting its requirement during the process. Notch signalling appears to act downstream from BMP signalling (Beck et al., 2003). Recent loss-of-function studies point to a similar role of Wnt/β-catenin signalling for blastemal formation during limb (Kawakami et al., 2006; Yokoyama et al., 2007) as well as fin regeneration (Poss et al., 2000). Moreover, reduced Wnt/β-catenin signalling abolishes FGF8, but not FGF10 expression, suggesting that it acts upstream of FGF8 and downstream from, or in parallel with FGF10 (Yokoyama et al., 2007).

Furthermore, innervation is known to be imperative for regeneration to occur. Nerve derived signals up-regulate genes important for the regenerative process, of which FGF2 is considered to be the most significant factor as it can rescue regeneration in denervated appendages (Mullen et al., 1996). It has been shown that innervation is required for maintenance of expression of genes in the early blastema of the froglet, including tbx5 and prx1, and for initiation of expression of msx1, FGF8 and FGF10 expression levels are also reduced in the denervated blastema of the axolotl (Christensen et al., 2001), suggesting that the requirement of neuronal input for FGF expression is a conserved feature among species. The anterior gradient protein family member nAG is a secreted ligand for Prod 1 and acts through it to promote cell division. The local expression of nAG after electroporation is sufficient to rescue a denervated blastema by acting directly on blastemal cells to stimulate their proliferation, thus pointing towards the classical nerve dependent growth of the early regenerate (Kumar et al., 2007; Kumar et al., 2010). The neurotransmitter substance P and
the iron-binding protein transferrin are also neural factors that have a positive effect on blastema cell proliferation (Nye et al., 2003).

**Morphogenesis and tissue repatterning**

The ability of cells to determine their position in three dimensions is crucial to the establishment of proper patterning in a developing or regenerating organ. Interactions with position-specific fibroblasts derived from the blastema are thought to provide a second signal that guides the developing and differentiating cells into the particular pattern needed to produce the completely regenerated structure by forming a type of connective tissue scaffold (Endo et al., 2004). Still it remains unclear as to what extent the newly generated cells inherit a particular positional identity from their differentiated precursors or rely on extrinsic cues from the cellular environment (Brockes, 1997). Classical experiments show that retinoic acid (RA) instructs positional identity in regenerating anuran tadpole or urodele limbs (Niazi and Saxena, 1978; Maden, 1982). RA affects the patterning of the proximodistal as well as dorsoventral axis of the regenerating axolotl limb (Ludolph et al., 1990) and also instructs bone patterning during outgrowth of regenerating fin (White et al., 1994). Endogenously, it is thought that the role of RA is to specify proximal identities by acting through the GPI (glycosylphosphatidylinositol) anchored cell surface molecule Prod1 (proximodistal-1) (da Silva et al., 2002) and through meis1 and meis2, two homeobox genes that are RA targets during limb development (Mercader et al., 2000) as well as in limb regeneration (Mercader et al., 2005). The newt homolog of CD59, Prod1 is a critical determinant of proximodistal identity for the limb blastemal cells during salamander limb regeneration (da Silva et al., 2002). Another factor, shh (sonic hedgehog) is important for imparting anterior-posterior axis information to the regenerating limb (Riddle et al., 1993; Roy et al., 2000; Roy and Gardiner, 2002). Interestingly shh is required for dorsoventral patterning of the regenerating spinal cord and also for regeneration of surrounding mesodermal tissues during Xenopus tail regeneration (Schnapp et al., 2005). The transcription factor Lef1 (lymphoid enhancer-binding factor 1) has a role in scleroblast alignment analogous to that proposed for shh during zebrafish fin regeneration (Poss et al., 2000).

Regenerative processes need to be tightly regulated to avoid overgrowth, mispatterning, and tumour formation. A few signals that negatively regulate regeneration have been identified. These are interesting from a therapeutic standpoint. Wnt5, likely activating a β-catenin independent signalling pathway inhibits zebrafish fin regeneration to aid in regrowing proper size of fin (Stoick-Cooper et al., 2007a). Myostatin is a highly specific muscle growth inhibitor and mice lacking myostatin display improved skeletal muscle regeneration (McCroskery et al., 2005)
FIBROBLAST GROWTH FACTORS

Many growth factors are known to play role during epimorphic regeneration *viz.* epidermal growth factor, fibroblast growth factors, transforming growth factors, nerve growth factor, platelet derived growth factor, vascular endothelial growth factor, etc. (Pilo and Suresh, 1994). Among the various growth factors involved in epimorphic regeneration, the role of Fibroblast growth factors (FGFs) has been studied at length in amphibians and also in several other regeneration models.

FGF was found in pituitary extracts by Armelin in 1973 (Armelin, 1973). Subsequently, it was found in a cow brain extract by Gospodarowicz and colleagues. They tested its activity in a bioassay which caused fibroblasts to proliferate (Gospodarowicz, 1974). FGFs consist of a family of twenty three members (FGF1 to FGF23), each consisting of a conserved core region of about 155 amino acids (Tanaka *et al.*, 2004). They are multifunctional proteins with a wide variety of effects; they are most commonly mitogens but also have regulatory, morphological, and endocrine effects. They have been alternately referred to as “pleuripotent growth factors and as “promiscuous growth factors” due to their multiple actions on multiple cell types (Vlodavsky *et al.*, 1990; Green *et al.*, 1996).

The functions of FGFs in developmental processes include mesoderm induction, antero-posterior patterning, limb formation, neural induction and brain development, and in mature tissues / systems angiogenesis, keratinocyte organization, and wound healing processes. FGF is critical during normal development of both vertebrates and invertebrates and any irregularities in their function leads to a range of developmental defects. FGFs also stimulate cells to migrate chemotactically (Clyman *et al.*, 1994; Sa and Fox., 1994; Landgren *et al.*, 1998). This is of importance both in angiogenesis and in wound healing (Burgess and Maciag, 1989). Further, FGFs stimulate cells to secrete proteases such as plasminogen activator (Mignatti *et al.*, 1989; Rusnati *et al.*, 1997; Miralles *et al.*, 1998), collagenase (Mignatti *et al.*, 1989; Hurley *et al.*, 1995; Aho *et al.*, 1997; Kennedy *et al.*, 1997; Newberry *et al.*, 1997) and gelatinase (Weston and Weeks, 1996). 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. FGFs also influence cell differentiation, stimulating the process in some cell types (Robinson *et al.*, 1995; Williams *et al.*, 1995; Kanda *et al.*, 1997) while inhibiting in others (Rapraeger *et al.*, 1991; Olwin and Rapraeger, 1992). Moreover, FGFs can also protect cells from undergoing apoptosis (Hughes *et al.*, 1993; Chow *et al.*, 1995; Guillonneau *et al.*, 1997).

FGFs are known to play significant roles in epimorphic regeneration as well. During blastema formation, the WE in both urodeles and larval anurans begins to express FGF8 (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* and results 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; Kostakopoulou et al., 1996). FGF1 is also known to influence blastemal cell proliferation during amphibian limb regeneration (Zenjari et al., 1996).

**FGF RECEPTORS (FGFRs) AND FGF SIGNAL TRANSDUCTION**

The biological effects of FGF are established as a result of intracellular signal transduction initiated by the growth factor-bound, activated FGF receptors (FGFRs). Four major receptor families have been identified FGFR1, FGFR 2, FGFR 3, and FGFR 4 (Basilico and Moscatelli, 1992; Jaye et al., 1992).

These receptors share common features including a cytoplasmic conserved tyrosine kinase domain, a transmembrane domain, and an extracellular ligand binding domain. However, spliced variants do exist that differ in the composition of the extracellular ligand binding domain, which can contain two or three immunoglobulin (Ig)-like loops (Hou et al., 1991; McKeehan et al., 1993). A single transmembrane stretch connects the extracellular part, with the intracellular juxtamembrane (JM) domain. The JM domains of FGFR1 and 2 contain one phosphorylatable tyrosine residue. FGFR3 and 4 lack tyrosine residues in their JM domains. The tyrosine kinase domain is split in two parts by a short non-catalytic insert of about 15 amino acid residues, which contains two phosphorylatable tyrosine residues in FGFR1 and FGFR2, one in FGFR3 and none in FGFR4. The mitogenic potential appears to be lower for FGFR4 than the other FGF receptors, which in part is due to the lack of the kinase insert tyrosine residues. The kinase insert tyrosine residues appear however to be dispensable for FGFR1 function. The C-terminal tails of the FGFRs contain a number of tyrosine residues of which some are located at identical positions in the receptors (Mohammadi et al., 1996; Wang and Goldfarb, 1997; Klint and Claesson-Welsh, 1999). A schematic structure of FGFR1 is shown in Figure I (A). The FGF receptor families are well conserved because FGFR1 to FGFR4 have been identified in species as primitive as *Drosophila*, *C. elegans* and Medaka fish (Emori et al., 1992).
Figure I. Schematic structure of FGFR-1. (A) The overall structural organization is similar for the four FGF receptors. The extracellular domain (EC) contains two (II and III) or three (I, II, III) immunoglobulin (Ig)-like domains, followed by the transmembrane (TM) stretch, the juxtamembrane (JM) domain, the kinase domain (KD) interrupted by a short kinase insert, and a C-terminal tail (CT). The acidic box indicated in the intracellular domain is a specific feature of FGF receptors. (B) The phosphorylatable tyrosine residues are indicated in the ligand-bound, dimerized FGFR-1 (Adapted from Klint and Claesson-Welsh, 1999).

Binding of FGF leads to dimerization of FGF receptors, which may be homodimers or heterodimers. FGFs are monomeric factors, although there are reports on the presence of two distinct receptor-binding sites in FGF, which might facilitate receptor dimerization. Dimerization of receptor tyrosine kinases appears to be a prerequisite for activation of the tyrosine kinase. Receptor activation leads to tyrosine autophosphorylation of the receptors (Figure I (B)). Tyrosine phosphorylation sites serve as high affinity binding sites for Src Homology2 (SH2) domain containing signal transduction molecules. These molecules transduce signals from the receptor in signalling chains or cascades, which eventually results in biological responses, often involving changes in gene transcription (Pawson, 1995; Ornitz et al., 1996; Klint and Claesson-Welsh, 1999). The FGFs differ in their abilities to signal through the different FGF receptor variants, which is an essential mechanism for regulating the specificity of FGF-induced downstream signalling and biological activities (Eswarakumar et al., 2005).

Heparan sulphate proteoglycans (HSPGs), key components of cell surfaces and extracellular matrices (ECM) are able to modulate different growth factor activities. In this context, cell-surface HSPGs bind soluble ligands, increasing their local concentration and modulating ligand-receptor encounters (Bernfield et al., 1999). In particular, FGF2 completely depends
on heparan sulphate to transduce an intracellular signal through its receptors (FGFRs) (Rapraeger et al., 1991; Yayon et al., 1991; Mansukhani et al., 1992) through the formation of the ternary complex HSPG-FGF2-FGFR (Pellegrini, 2001). Syndecans and glypicans are the two families of HSPGs that localize to the plasma membrane. In different systems, it has been shown that syndecans (Bernfield and Sanderson, 1990; Chernousov and Carey, 1993; Filla et al., 1998; Fuentealba et al., 1999; Villena et al., 2003; Zhang et al., 2003) and glypicans (Song et al., 1997; Midorikawa et al., 2003; Su et al., 2006) have the ability to bind FGF2, modulating its binding and signalling.

**FIBROBLAST GROWTH FACTOR 2**

FGF2 was initially identified as a 15 kDa protein (Gospodarowicz, 1975), that was later found to represent a proteolytic product of the primary 18kDa form (Bikfalvi et al., 1997). Different protein isoforms of FGF2 result from alternative translational initiation, giving rise to 21- to 24-kDa forms (collectively referred to as high-molecular-weight [HMW] isoforms) with limited tissue distribution and to the ubiquitously expressed 18-kDa form (Ornitz and Itoh, 2001). The high molecular weight (HMW) forms contain the complete low molecular weight (LMW) sequence in addition to an NH₂-terminal extension of varying lengths (Moscatelli et al., 1987; Sommer et al., 1987; Florkiewicz and Sommer, 1989). HMW isoforms, on the other hand, remain intracellular and appear to elicit different biological functions - including migration, proliferation, and transformation - than the 18-kDa isoform does. These functions are both dose as well as cell type dependent (Chandler et al., 1999; Ornitz and Itoh, 2001). One of the most striking features of FGF2 is the lack of a consensus signal sequence for secretion. While significant amounts of the 18kDa form of FGF2 are found outside the cell, the higher molecular weight forms are predominantly localized to the nucleus (Bikfalvi et al., 1997). The nuclear targeting of the HMW forms seems to result from the amino-terminal extensions which contain several Gly-Arg repeats with methylated Arg residues (Bikfalvi et al., 1997).

The biological activity of the 18-kDa FGF2 requires the presence of both FGF receptors (FGFRs) and HSPGs localized at the cell surface (Ornitz et al., 1992; Guillonneau et al., 1996; Ornitz and Itoh, 2001). Initially, the association of FGF2 with heparan sulfate has been proposed to protect this FGF from proteolysis and thermal denaturation (Saksela and Rifkin, 1990; Vlodavsky et al., 1996) and to serve as a reservoir of growth factor that can be released by enzymes that degrade the proteoglycans (Saksela and Rifkin, 1990). Later, HSPGs were identified as co-receptors for FGF2, strongly promoting FGF-FGFR binding and the subsequent activation of the receptor (Steinfeld et al., 1996).

Cells that do not express HSPG show reduced receptor binding affinity and reduced biological response (Conrad, 1998). The addition of heparan, heparan sulphate or HSPG can
replace the function of cell associated HSPGs by enhancing FGF2 receptor binding under some conditions and inhibiting under others. The reason why heparan and heparan sulphate both stimulate and inhibit FGF2 receptor binding and activity is likely based on a combination of the chemical structure of the glycosaminoglycan chains and the physical localization of the HSPG relative to the cell surface. A possible mechanism for inhibition might involve sequestration of FGF2 by HSPG within the extracellular matrix such that receptor binding and activation is inhibited. Cell associated HSPGs could potentiate activity by associating with FGF2 and its receptors. The eventual response (stimulation vs. inhibition) elicited by HSPG would relate to the relative concentrations and binding kinetics for FGF2 of the various pools of HSPG. The type of cellular response (i.e. proliferation, migration, differentiation) might depend on the specific cell surface HSPG and FGF receptor type expressed. The specific core protein (i.e. syndecans, perlecan, glypicans) and cell as well as tissue specific differences in heparan sulphate modification result in altered FGF2 regulation (Conrad, 1998).

The mechanism of secretion of the 18kDa FGF2 remains unclear. FGF2 does not progress through the endoplasmic reticulum and the Golgi via the regular secretory pathway. It has been suggested that FGF2 is released from the cells as the result of cell damage, death and non-lethal membrane disruptions (Conrad, 1998). Released FGF2 is found stored in the extracellular matrix and basement membranes bound to HSPG (Folkman et al., 1988). The movement and distribution of FGF2 within the extracellular matrix is controlled by diffusion with rapid reversible binding to HSPG (Dowd et al., 1999). Alterations in FGF2-HSPG interactions by proteolytic degradation of HSPG, competitive antagonists, soluble heparan and analogs can dramatically enhance FGF2 release from extracellular matrix sites (Bikfalvi et al., 1997; Dowd et al., 1999). Extracellular FGF2 binds to cell surface receptors and HSPGs and is subject to internalization and lysosomal degradation. However, a considerable amount of FGF2 can translocate into the nuclear fraction of various cell types (Sperinde and Nugent, 1998). Nuclear translocation is cell-cycle dependent, occurring in the G1-S transition. This results in an overall decrease in FGF2 degradation and correlates with enhanced mitogenic activity (Bikfalvi et al., 1997; Conrad, 1998; Sperinde and Nugent, 1998). HSPGs cause direct FGF2 internalization, presumably as a result of constitutive HSPG internalization and turnover (Conrad, 1998). FGF2 can also be internalized in HSPG/receptor ternary complexes, yet the rate of internalization by this route appears to be the same as that via receptors alone (Fannon and Nugent, 1996). Indeed, it has been shown that HSPGs accelerate nuclear localization, increase cytoplasmic uptake and inhibit degradation of FGF2 in vascular smooth muscle cells (Sperinde and Nugent, 1998). Thus FGF2 appears to be subject to several distinct fates within cells depending on whether complexed to a receptor, HSPG or both.
FGFR1 and FGFR2 bind FGF2 with the greatest affinity, but the level of redundancy in receptor utilization within the FGF family is high (Ornitz et al., 1992). In spite of this redundancy, targeted gene-inactivation of different FGF members yields a specific phenotype for each factor. The major signalling cascades activated by FGF2 following receptor phosphorylation and activation are shown in Figure II.

**Figure II.** FGF2 has been shown to activate a number of intracellular signalling pathways. Major, well characterized processes that have been identified in a number of cell types are shown. The binding of FGF2 to its receptors is enhanced by cell surface HSPG and leads to activation of autophosphorylation of the FGFR on several tyrosine residues. Some of the phosphotyrosine residues are binding sites for src homology domain containing proteins such as phospholipase C-γ and others are binding sites for proteins with phosphotyrosine-binding domains such as FGF receptor substrate 2 (FRS2) and SHC. SHC and FRS2 function as docking proteins those bind to the GRB2-SOS complex which then activates RAS. RAS recruits RAF-1, a serine/threonine kinase that activates MEK. MEK proceeds to activate the mitogen activated protein kinases (MAPK) which translocate to the nucleus where they directly activate transcription factors by phosphorylation. The activation of PLC-γ also plays a major role in transmitting the eventual FGF2-mediated biological signals. PLC-γ activation results in hydrolysis of phosphatidylinositol to inositol-3-phosphate and diacylglycerol (DAG) leading to Ca²⁺ release and activation of protein kinase C (PKC). The specific signalling pathways and molecules involved can depend on the particular FGFR type activated. Furthermore, signalling directly from cell surface HSPGs, as well as direct actions of intracellular and nuclear FGF2 might also be important in determining the cellular response. As a result of this complexity, FGF2 activation of various cell types can lead to a number of end-point biological responses (Adapted from Nugent and Iozzo, 2000).
FGF2 has pleiotropic effects in different cell and organ systems. In contrast to other FGFs that have a restricted pattern of expression, FGF2 is present in the majority of tissues of both adult and embryonic origin, and it is produced by many cell types. An overwhelming variety of pharmacological effects have been reported for FGF2, both in vitro and in vivo (Bikfalvi et al., 1997). FGF2 acts as a mesoderm inducer when applied to Xenopus embryonic caps, and it can substitute the apical ectodermal ridge and maintain proliferation of limb bud mesenchyme during limb development (Slack et al., 1987; Fallon et al., 1994). FGF2 is a potent chemotactic factor for fibroblasts and endothelial cells, can promote or inhibit cell differentiation, and is a potent angiogenic and neurotrophic factor (Bikfalvi et al., 1997). It promotes differentiation of both endothelial cells and hematopoietic cells from dissociated quail epiblasts, which, along with its angiogenic activity, suggest a role for FGF2 in blood vessel development (Flamme and Risau, 1992). In the hematopoietic system, FGF2 enhances myelopoiesis in long-term bone marrow cultures and is a potent stimulator of megakaryocytopenesis (Wilson et al., 1991; Avraham et al., 1994). The activities of FGF2 in the central nervous system are also multiple. FGF2 maintains survival of isolated neurons, promotes neurite outgrowth of hippocampal and cortical neurons (Matsuda et al., 1990) and regulates expression of neurotransmitters like neuropeptide Y (Barnes and Cho, 1993). FGF2 stimulates division of cortical multipotent stem cells and may also act on postmitotic neurons to promote differentiation and survival (Ghosh and Greenberg, 1995; Temple and Qian, 1995; Qian et al., 1997). FGF2 promotes quiescent astrocytes to re-enter the cell cycle and induces expression of glial fibrillary acidic protein, a marker of astrocyte differentiation (Kniss and Burry, 1988). FGF2 acts as a survival factor in many models of cell and tissue injury. Topical application of FGF2 accelerates healing of skin wounds in animal models, as well as of eye, retina and corneal wounds (Bikfalvi et al., 1997).

Besides its many roles in several physiological and developmental processes, FGF2 is also one of the key players of epimorphic regeneration. FGF2 has been localized to the WE and nerves of the regenerating amphibian limb and it can re-establish the expression of several genes, which had been inactivated after denervation, thus allowing denervated limbs to regenerate (Mullen et al., 1996). FGF2, in addition to being up-regulated in the regenerating spinal cord in newts, is also expressed in a subset of blastemal cells and chondroblasts, in the basal epidermal layer and also in differentiating muscle (Ferretti et al., 2001). FGF2 soaked beads can stimulate chick limbs, which normally do not regenerate, to do so (Taylor et al., 1994; Kostakopoulou et al., 1996). Implantation of FGF2 soaked beads can even induce extra limbs from the flank of chick embryo in vivo (Cohn et al., 1995). Furthermore, FGF2 is known to promote blastemal growth during zebrafish fin regeneration as well (Hata et al., 1998).
Since FGF2 is implicated in a variety of growth disorders and cancers because of its role in developmental events as well as due to potent mitogenic activity, it seems reasonable that blocking the FGF2 signal activity via inhibition of the tyrosine activity of its receptor would be of therapeutic value. Indolinones are polycyclic compounds that bind the ATP binding pocket of receptor tyrosine kinases, inhibiting their activities. The pharmacological inhibitor SU5402 (Figure III) is one such indolinone that inhibits the tyrosine kinase activity of FGFR1 by interacting with its catalytic domain. It acts only as a weak inhibitor of tyrosine phosphorylation of the PDGF (Platelet Derived Growth Factor) receptor, does not inhibit the phosphorylation of insulin receptor and exhibits no inhibitory effect on EGF (Epidermal Growth Factor) receptor kinase (Mohammadi et al., 1997). The various FGFR1 isoforms have different affinities for FGFs, however, the only FGF that FGFR1 binds with high affinity are FGF1 and FGF2. However, FGF1 downstream signalling can occur via binding to all FGFRs (Zhang et al., 2006). Hence, an increasing number of studies have targeted the FGF2 pathway through inhibition of the tyrosine kinase activity of the fibroblast growth factor receptor 1 by use of SU5402 (Mohammadi et al., 1997; Poss et al., 2000; Smith et al., 2005; Mori et al., 2007; Izikki et al., 2009; Woad et al., 2009; Edel et al., 2010, Lamont et al., 2011). Hence, in the current study use of SU5402 was done to block FGFR1 and to understand the role of FGF2 signalling in the process of reptilian epimorphosis.

**Figure III.** SU5402 (3-[3-(2-Carboxyethyl)-4-methylpyrrol-2-methylidenyl]-2-indolinone)
LIZARD TAIL AS A MODEL TO STUDY EPIMORPHIC REGENERATION

The voluntary shedding of an expendable body part, or autotomy (self amputation), is a defensive strategy for last-minute escape from predation that has evolved independently in a wide range of organisms (Juanes and Smith, 1995; Souter et al., 1997; Shargal et al., 1999; Bernardo and Agosta, 2005). It is well understood that in many lizard groups such as geckos, scincids and lacertids, loss of the tail (typically by autotomy) results in an open wound. This wound site undergoes scarless healing and, ultimately, regeneration of a functionally equivalent structure (Alibardi, 2010).

To date, most research on naturally evolved epimorphic regeneration in vertebrates has focussed on non-amniotes including teleosts (e.g. zebrafish) and urodeles (e.g. axolotls and newts) (Brockes and Kumar, 2008). But such studies for reptilian system have been neglected despite the fact that the lizard represents the best non mammalian amniote to analyze the molecular factors involved in the regeneration of various tissues in the tail. In fact, many of the events observed during epimorphic tail regeneration in lizards are conserved with those of urodeles and teleosts like wound repair, blastema proliferation and tissue morphogenesis (Alibardi, 2010).

Although, detailed cytological information on the process of tail and limb regeneration in lizards is now available (Alibardi, 2010), there is little molecular information on specific genes and proteins activated during regeneration. At our department several aspects of regeneration have been explored in lizards. Studies on the histological, biochemical and metabolic alterations in gekkonid lizard Hemidactylus flaviviridis during regeneration have been carried out (Kumar and Pilo, 1994; Pilo and Suresh, 1994; Pilo and Kumar, 1995; Yadav et al., 2012). Recently, studies also showed that prostaglandin E2, one of the inflammatory mediators positively regulates H. flaviviridis tail regeneration (Sharma and Suresh, 2008; Suresh et al., 2009). Since, evolutionarily, reptiles are closer to mammals than amphibians, an in-depth understanding of the finer mechanisms of regeneration in reptiles is more significant as this knowledge can be better extrapolated and applied to mammalian system which has got only limited regenerative ability.

Increasing evidences are now available from research on appendage regeneration in urodele that FGF2 is one of the prime modulators of epimorphic regeneration. Hence, it was thought pertinent to study its influence if any, on reptilian epimorphosis. Initial work done in our lab proved beyond doubt that FGF2 influences H. flaviviridis tail regeneration positively. It was apparent from the morphometric analysis that FGF2 significantly influences the wound epithelial (WE) and blastemal stages of tail regeneration in H. flaviviridis. These findings were further confirmed by administering anti-FGF2 and following the progress of tail
regeneration (Yadav, 2005). Hence, the current study was undertaken to elucidate how FGF2 modulate the caudal regeneration in *H. flaviviridis* by analysing the cardinal molecular processes of regeneration.

**AIM OF THE STUDY**

The process of epimorphosis involves several events like programmed cell death, matrix reorganization, cell migration leading to the formation of a functional wound epithelium, subsequently followed by proliferative activities and differentiation which eventually restore the lost part. **The present study was aimed at understanding the involvement of FGF2 signalling in achieving several quintessential milestones of epimorphic regeneration using *H. flaviviridis* as animal model.** This was fulfilled by studying the following objectives.

First, to signify the role of FGF2 during tail regeneration of *H. flaviviridis*, its immunohistochemical localization was done during the key stages of regeneration viz. wound epithelium, blastema and differentiation stages in the normal regenerating tail. Its levels were also quantified at these regenerative stages as well as in resting state by ELISA. An idea of the changing levels of FGF2 during regeneration and the target cells/ tissues of FGF2 could be gained from this spatial and temporal analysis.

Thereafter, to establish the importance of FGF2 in reptilian regeneration, the FGF2 signalling pathway was targeted via inhibition of tyrosine kinase activity of the FGF receptor 1 (FGFR1) using the pharmacological inhibitor SU5402 and the effects on several important events of regeneration were studied. Initially, the effect of FGF2 signalling inhibition on various stages of regeneration was observed through a basic morphometric analysis. The duration for control and SU5402 treated groups to achieve defined stages of regeneration was recorded and the growth rate calculated. In support of the morphological observations, further, a histological study of the regenerates was done to understand the effect of impaired FGF2 signalling on the tissue architecture (Chapter 1).

After amputation, the first stage during epimorphic regeneration is wound healing and formation of a functional wound epithelium, during which, one of the important events is matrix reorganization. Since, FGF2 is known to accelerate formation of wound epithelium (Maher et al., 2001; Yadav et al., 2012), it was important to find out its role in matrix remodelling. This was achieved by analysing the enzymes associated with it like Matrix metalloproteinases (MMP-2, 9) through Immunohistochemistry and Gelatin Zymography. Further, MMP degradative activity is tightly regulated by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs), since excess proteolytic activity can be detrimental to regeneration. Hence, by studying the levels of TIMP, one can analyze the role of FGF2 in the
interplay between MMP and TIMP and the process of matrix reorganization. TIMPs were assessed through reverse gelatin zymography. Further, biosynthesis of proteins is one of the most important biochemical processes during regeneration. Hence, a protein profiling for the regenerates was also done through SDS-PAGE to understand the protein turnover during regeneration (Chapter 2).

During the initial matrix remodelling activities, one of the accompanying events is cell apoptosis, both of which are equally important for the successful formation of the wound epithelium. In fact, apoptosis is required during early stages of tail regeneration in *X. laevis* and inhibition of caspase-3 activity during these stages leads to inhibition of the regenerative process (Tseng *et al*., 2007). FGF2 (along with bone morphogenetic proteins) is known to be involved in apoptosis as well as proliferative activities and a controlled regulation of these factors and the associated processes is required for eventual regeneration. Thus to understand, how FGF2 influences apoptosis in regenerating tail of *H. flaviviridis*, TUNEL staining for the regenerates as well as immunolocalization of Caspase-3 was done. Further, formation of the blastema and its subsequent differentiation requires cell proliferation at a high rate. This is accompanied by rapid angiogenesis, which is a precondition for cell proliferation. To gain an understanding of the influence of FGF2 on these proliferative activities, BrdU incorporation followed by immunolocalization of the BrdU positive cells in the tail regenerates was done. In support of this notion, histofluorescence localization of nucleic acids through acridine orange staining of tissue regenerates during the proliferative stages was also carried out. Moreover, angiogenesis at the site of injury is an important process that determines the quality of repair. Hence, localization for the angiogenic molecule VEGF (Vascular Endothelial Growth Factor) was carried out to know how FGF2 influences angiogenesis during reptilian epimorphosis. Further, Angiogenesis can be traced by estimating COX-2 levels, which acts as an inducer of angiogenesis factors and also inhibits apoptosis (Kurie and Dubois, 2001). COX-2 is also an upstream modulator of PGE2, one of the inflammatory mediators involved in regeneration. Some theories suggest that FGF2 works through COX-2 and prostaglandin pathway (Bikfalvi *et al*., 1997; Foegh and Ramwell, 2004) whereas others point that COX-2 mediates FGF2 signalling (Finetti *et al*., 2008). Finally therefore, a localization study for COX-2 in FGF2 signal inhibited animals and of FGF2 in COX-2 inhibited animals was done to understand the interplay between these two important regulators of epimorphosis (Chapter 3).