# ROLE OF FIBROBLAST GROWTH FACTOR 2 (FGF2) IN THE INITIATION AND PROGRESSION OF TAIL REGENERATION IN NORTHERN HOUSE GECKO *HEMIDACTYLUS FLAVIVIRIDIS*

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## CERTIFICATE

This is to certify that the thesis "**ROLE OF FIBROBLAST GROWTH FACTOR 2 (FGF2) IN THE INITIATION AND PROGRESSION OF TAIL REGENERATION IN NORTHERN HOUSE GECKO *HEMIDACTYLUS FLAVIVIRIDIS***" incorporate the results of investigation carried out by the candidate herself and analyzed in the Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara.

Candidate
(Pillai Anusree Narayanan)

Guiding Teacher
(Dr. B. Suresh)

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*Anusree Pillai*

# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| AEC | – | Apical epithelial cap |
| ALP | – | Alkaline phosphatase |
| AO | – | Acridine orange |
| BCA | – | Bicinchoninic acid |
| BCIP | – | 5-bromo-4-chloro-3-indolyl-phosphate |
| BL | – | Blastema |
| BrdU | – | Bromodeoxyuridine |
| BSA | – | Bovine serum albumin |
| COX-2 | – | Cyclooxygenase-2 |
| DAB | – | 3, 3’ Diaminobenzidine |
| DF | – | Differentiation |
| DMSO | – | Dimethyl sulphoxide |
| dpa | – | Days post amputation |
| ECM | – | Extra cellular matrix |
| ELISA | – | Enzyme linked immunosorbent assay |
| FGF2 | – | Fibroblast growth factor 2 |
| FGFR | – | Fibroblast growth factor receptor |
| FITC | – | Fluorescein isothiocyanate |
| hpa | – | Hours post amputation |
| HRP | – | Horse radish peroxidase |
| HSPG | – | Heparan sulphate proteoglycan |
| MMP | – | Matrix metalloproteinase |
| NBT | – | Nitro blue tetrazolium |
| OCT Medium | – | Optimal cutting temperature Medium |
| PBS | – | Phosphate buffered saline |
| PBST | – | Phosphate buffered saline-Tween-20 |
| $PGE_2$ | – | Prostaglandin $E_2$ |
| SDS-PAGE | – | Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis |
| TIMP | – | Tissue inhibitor of matrix metalloproteinase |
| TMB | – | Tetramethyl benzidine |
| TUNEL | – | Terminal deoxynucleotidyl transferase dUTP nick end labelling |
| VEGF | – | Vascular endothelial growth factor |
| VEGFR | – | Vascular endothelial growth factor receptor |
| WE | – | Wound epithelium |

# INTRODUCTION

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 18$^{th}$ 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 20$^{th}$ 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).

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 msx1and 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 and2, 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).

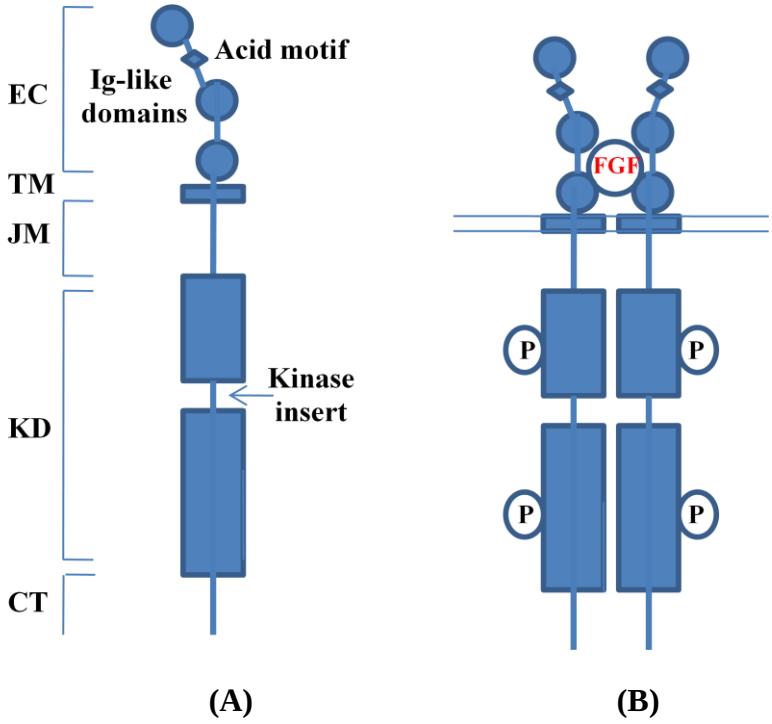



**Figure I.** Schematic structure of FGFR-1. **(A)** The overall structural organization is similar for the four FGF receptors. The extracellular domain (EC) contains two (II and III) or three (I, II, III) immunoglobulin (Ig)-like domains, followed by the transmembrane (TM) stretch, the juxtamembrane (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 $NH_2$-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.

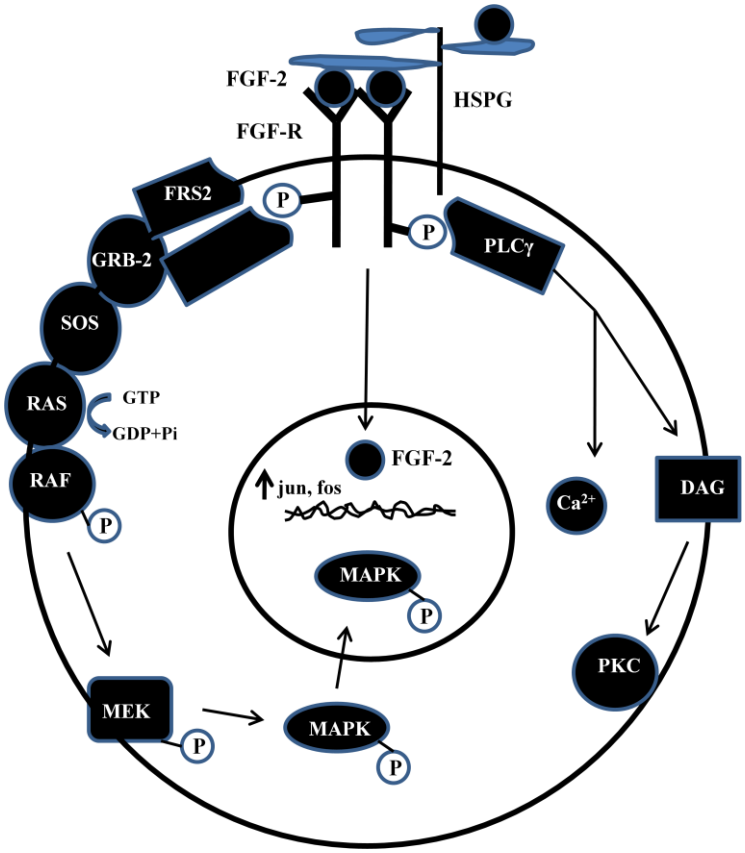



**Figure II.** FGF2 has been shown to activate a number of intracellular signalling pathways. Major, well characterized processes that have been identified in a number of cell types are shown. The binding of FGF2 to its receptors is enhanced by cell surface HSPG and leads to activation of autophosphorylation 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 $Ca^{2+}$ 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 megakaryocytopoiesis (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.

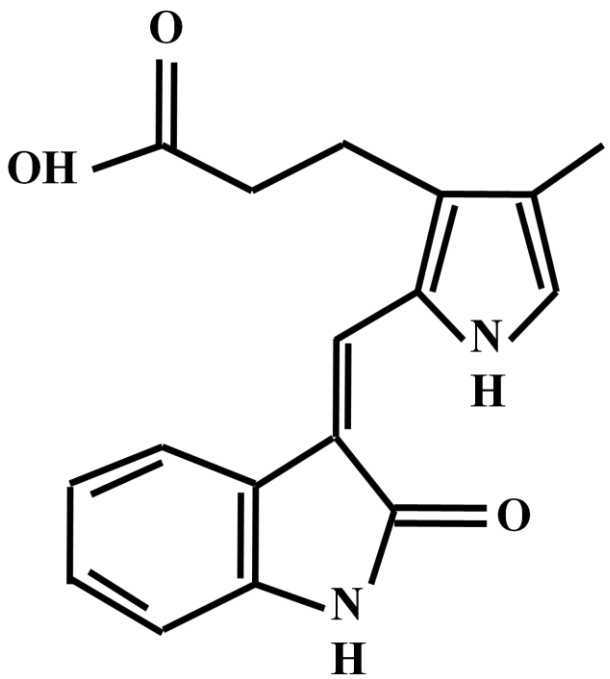



**Figure III.** SU5402 (3-[3-(2-Carboxyethyl)-4-methylpyrrol-2-methylidenyl]-2-indolinone)

## 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 regenration 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 $E_2$, 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).

# MATERIAL AND METHODS

## ANIMAL AND MAINTENANCE

Adult Northern House Gecko, *Hemidactylus flaviviridis* Rüppell, 1835, (both the sexes) with normal intact tails, weighing 10±2g, collected from natural habitat were procured from local animal dealer. They were housed in well ventilated wooden cages of 45x30x60 cm with glass slider on one side for light and visibility, in the Departmental animal house (827/ac/04/CPCSEA). The lizards were subjected to 12:12 hour light-dark cycles and room temperature was maintained at 30±2ºC. The relative humidity was between 30 and 70%. Further, experiments were conducted in the months of March-July and September-November. These environmental conditions are necessary to evoke optimal regeneration in lizards. All animals were screened for parasitic infestation and/or wounds and the healthy ones were acclimated for a week before the commencement of experiment. The animals were fed with cockroach nymphs twice a week and purified water was given daily, *ad libitum*.

## DRUGS AND DOSAGE

### Preparation of Drug solutions

1. SU5402: A stock solution of SU5402 was prepared in DMSO and stored at 4ºC. Prior to the start of an experiment, this stock was diluted to obtain a final dilution of SU5402 in 1% DMSO, which was used for treatment.
   Dosage: 0.7mg/kg body weight

2. Etoricoxib: The drug is soluble in alkaline pH and was prepared in Tris Buffer (pH 8.8) fresh before use.
   Dosage: 50mg/kg body weight

SU5402 was purchased from Calbiochem®, EMD Biosciences, Inc., USA and Etoricoxib was gifted by the manufacturer - Sunpharma Advanced research Company Ltd., Vadodara, India. All the other chemicals were of AR grade and were procured from SRL Pvt. Ltd., Mumbai, India and Qualigens fine chemicals, Mumbai, India.

### Drug Dosage and Route of Administration

For SU5402, dosage was selected based on an initial dose range study. In case of etoricoxib dosage was decided according to initial studies carried out in our laboratory (Sharma and Suresh, 2008). Animals were given *in loco* injections (at second intact tail segment from vent) at a maximum quantity of 0.075ml/animal.

## EXPERIMENTAL PROTOCOLS

### Method for induced autotomy

Autotomy was induced by exerting mild thumb pressure on the normal intact tail, two segments away from the vent. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) in accordance with the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India (Form B No. ZL/IAEC/13-2010). All protocols of amputation and treatment were done under hypothermic anaesthesia (Reilly, 2001). Irrespective of the experiments, sample size per group was at least five and has been duly exacted in the respective chapters.

### Treatment Schedule

For morphometric study, treatment was conducted in two series. In the first series, treatment started two days prior to amputation and was continued on every alternate day till the termination of the experiment (when control animals reached differentiation stage). In the second series, after inducing autotomy, drug treatment started at different stages of tail regeneration, *viz*., wound epithelium stage and blastema stage and was continued on every alternate day till the termination of the experiment, to study the stage specific effects. After amputation, the process of wound healing initiates leading to the formation of a wound epithelium, which appears as a smooth shining surface. Blastemal stage is characterized by conical aggregation of cells called blastema, a regenerate about 2-3mm in length. The blastema further grows in size and later on differentiates to replace the missing structures. For the rest of the experiments, the same treatment schedule as in the first series was followed. Regenerates were collected at defined stages and were processed further according to the experimental requirement.

### Morphometric measurements of tail growth

The growth of the regenerate was measured at fixed intervals using a calibrated digital Caliper (Mitutoyo, Kawasaki, Japan). The time taken to reach defined stages of regeneration such as wound epithelium, blastema and differentiation was recorded and growth rate of regenerate was calculated.

### Histological study of regenerates

Regenerates fixed in 10% formalin were washed thoroughly in running water, followed by decalcification in 10% nitric acid solution. The fixed tissue samples were then dehydrated by placing them in increasing concentrations of alcohol (50, 70 and 95%) and then in a 1:1 solution of alcohol and xylene, followed by placing them in xylene alone until the tissues became transparent. The tissues were then hot infiltrated with paraffin wax by first placing them in mixtures containing xylene and wax with successively increasing concentrations of

the wax (75:25, 50:50, 25:75 mixtures of xylene and wax respectively) and finally in molten paraffin wax alone for 1-2 hours. Wax blocks of the infiltrated tissues were prepared and longitudinal sections (7µm) of tail tissues were cut using a Leica microtome and placed on glass slides coated with egg albumin.

Slides were then exposed to xylene, dried and passed through decreasing order of alcohol (95, 70 and 50%) for few minutes, washed in distilled water and stained with Harris's haematoxylin for 2-3 minutes. The slides were then washed and stained with eosin for 1-2 minutes, dehydrated in increasing concentration of alcohol (70 and 95% for few seconds), dried and cleared in xylene before mounting permanently with DPX. The tissue sections were observed under a Leica DM2500 Microscope and pictures captured using EC3 Camera (utilizing LEICA LAS EZ (V 1.6.0) software).

**Immunofluorescence localization of FGF2, COX-2, VEGF and Caspase-3**

Briefly, regenerating tails at different stages were collected by inducing autotomy to release the regenerate along with an intact adjacent tail segment. These were embedded in optimal cutting temperature medium (Tissue-Tek OCT, Sakura Finetek, USA) and frozen at -20ºC until used for cryosectioning.

For immunolabelling, longitudinal cryosections (8-10µm) were fixed in acetone at -20ºC for 15-20 minutes and air dried for 15 minutes. Sections were then rehydrated with PBST (Phosphate Buffered Saline with 0.025% Tween-20) followed by blocking with corresponding normal serum [Genei, Merck, USA; 10% in PBS with 0.5% Bovine serum albumin (PBS-BSA)] for 1-2 hours at room temperature (RT). Sections were then incubated with appropriate primary antibody [1:200 dilution of Rabbit Anti-FGF2 (Sigma-Aldrich, USA) in PBS-BSA; 1:50 dilution of Goat Anti-COX-2 (Biolegend, USA) in PBS-BSA; 1:100 dilution of Rat Anti-VEGF (Biolegend, USA) in PBS-BSA or 1:200 dilution of Rabbit Anti-Caspase-3 (Sigma-Aldrich, USA) in PBS-BSA] overnight inside a moist chamber at 4ºC. Following day, sections were washed with PBST thrice for 5 minutes each and incubated with a corresponding FITC conjugated secondary antibody [1:50 dilution of Goat Anti-Rabbit IgG-FITC, Goat Anti-Rat IgG-FITC or Rabbit Anti-Goat IgG-FITC (Genei, Merck, USA) in PBS] for 2 hours at RT. Sections were then washed with PBS thrice for 5 minutes each and mounted in 1:1 mixture of PBS:glycerol and observed using a fluorescent microscope (Leica DM2500). Same procedure was followed for negative control sections except that these were incubated with PBS-BSA instead of the primary antibody.

**Immunohistochemical localization of MMP-2 and MMP-9**

As described before, regenerates along with an intact tail segment were collected at different stages of regeneration, embedded in OCT and fresh frozen longitudinal cryostat sections (8-

10µm) of these were taken on clean glass slides. These were fixed in cold acetone at -20ºC for 15-20 minutes, air dried for 15 minutes and rehydrated with PBST, then blocked using normal serum (10% in PBS-BSA) for 1-2 hours at RT. Sections were then incubated with appropriate primary antibody [1:200 dilution of Rabbit Anti-MMP-2 or Goat Anti-MMP-9 (Sigma-Aldrich, USA) in PBS-BSA] overnight inside a moist chamber at 4ºC. Next day, sections were washed with PBST (3x5min) and incubated with corresponding ALP-conjugated secondary antibodies [(Genei, Merck, USA)1:300 dilution in PBS] for 2 hours at RT, washed and stained with substrate chromogen solution (BCIP/NBT) (Genei, Merck, USA) for 10-15 minutes. Sections were washed with PBS, mounted with a solution of PBS and glycerol (1:1) and observed using a Leica DM2500 microscope. Negative control sections were incubated with PBS-BSA instead of the primary antibody.

**Estimation of Protein content**

Protein estimation of the tissue samples was done by a BCA (Bicinchoninic acid) assay kit (Genei, Merck, USA). This method, first described by Smith *et al*., 1985, combines the reduction of $Cu^{2+}$ to $Cu^{+}$ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation ($Cu^{+}$) using a reagent containing BCA. The purple coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. It is water soluble and exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range. The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for colour formation with BCA (Wiechelman *et al*., 1988). The amount of protein present in a solution can be quantified by measuring the absorption spectra and comparing with protein solutions with known concentrations.

Briefly, a set of BSA protein standards ranging from 0-2mg/ml was prepared. 20µl of each standard replicate was added to a microplate well, to which 200µl of BCA reagent was added. Same procedure was followed for unknown samples. This was allowed to incubate for 30 minutes at 37ºC, cooled to room temp and absorbance was read at 562nm on a plate reader (Metertech ∑960). A standard curve of absorbance versus concentration of BSA was plotted and a regression equation was calculated in ms excel to determine the amount of protein in the unknown samples.

This assay was carried out to use equal amounts of protein for FGF2 quantification, zymographic studies and SDS-PAGE analysis.

**FGF2 Quantification by ELISA**

FGF2 level in the regenerates was quantified using an ELISA Kit (Quantikine, R&D Systems, USA) based on a quantitative sandwich enzyme immunoassay. In brief, a set of FGF2 standards (0-1000pg/ml) was prepared and taken in replicates onto microplate wells precoated with FGF2 specific antibody. Similarly, unknown samples with equal protein concentration were also loaded onto the microplate. Standard and unknown samples were appropriately diluted with diluent buffer, the plate covered with foil and incubated for 2 hours at room temperature. The wells were then aspirated, washed thrice with the wash buffer and blotted dry by inverting on clean tissue paper. Next, biotin-conjugated antibody solution specific for FGF2 was added and incubated for 2 hours at room temperature, followed by aspiration and washing thrice. Next, incubation with Streptavidin-HRP solution was done for 2 hours at room temperature and plate was again washed thrice. Finally, substrate-chromogen solution (TMB-$H_2O_2$) was added and plate kept in dark for 30 minutes. The reaction was stopped with the stop solution and the colour change was measured at a wavelength of 450nm using an ELISA plate reader (Metertech ∑960). FGF2 concentration in the unknown samples was determined by comparing/computing the results with the standard curve/regression equation.

**SDS-PAGE (Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis) Analysis of Proteins**

This method is used for electrophoretic separation of proteins through a gel matrix formed of polyacrylamide chains cross linked by N,N-methylene bisacrylamide. Since the proteins in their native or partially denatured form vary in their charge to mass ratio, SDS, an anionic detergent is used to denature proteins to their primary (linearized) structure and coat them with uniform negative charge as one SDS molecule binds to 2 amino acids. Thus, the charge to mass ratio of all the denatured proteins in the mixture becomes constant and protein molecules are separated according to their molecular weights only. The resolution and focus of the protein bands is increased by using a discontinuous gel system (Laemmli, 1970) using Tris-Glycine that stacks at a pH of 6.8 and resolves at a pH of 8.8. A standard is run along with the sample to analyze the separated proteins

Briefly, the procedure consists of the following steps:

1. Making the polyacrylamide gel using a Gel assembling apparatus. 2. Preparing the samples by boiling in sample loading buffer containing SDS, glycerol and a reducing agent 3. Loading equal protein amounts on the gel and running the electrophoresis 4. Staining of the proteins using Coomassie Brilliant Blue-R250.

**Zymography analyses**

Zymography and reverse zymography are techniques used to evaluate the activities of MMPs and TIMPs in biological samples. Proteins are electrophoretically separated using a polyacrylamide gel containing a co-polymerized specific substrate under denaturing (SDS), non reducing conditions. After exchange of SDS with Triton X-100, enzymes partially renature and recover their activity. Subsequently, during incubation of gel in an appropriate activation buffer, MMPs in the gel digest the substrate. These digested areas of MMP activity are seen as clear areas against a dark background of undegraded substrate upon staining. Gelatin zymography involves using gelatin as the substrate and is mainly done for the detection of the gelatinases MMP-2 and MMP-9. In reverse zymography, an altered zymography method, an MMP is also included into the gel along with gelatin, usually MMP-2. During activation, MMP-2 digests gelatin in areas where TIMPs are absent. Thus, except for the TIMP bands where MMP activity will be inhibited, the remaining gel will appear colourless upon staining (Hawkes *et al*., 2001).

Gelatin Zymography

Briefly 7.5% SDS polyacrylamide gels with gelatin (5mg/ml) were prepared. Samples were loaded using a non reducing loading buffer. After electrophoresis, gels were washed with 2.5% Triton X-100 (2x30 min) and rinsed in double-distilled water ($ddH_2O$) followed by wash in incubation buffer (50mM Tris, 0.2M NaCl, 5mM $CaCl_2$, 0.02% $NaN_3$ and 0.02% Brij-35, pH 7.4-7.6) for 30 minutes and an 18 hour incubation with the same at 37ºC. Gels were stained with Coomassie Brilliant Blue-R250 (Coomassie 0.5%w/v, Methanol 40%v/v, Acetic acid 10%v/v in $ddH_2O$) for 1-2 hours and then destained ( 45:10:45 Methanol:Acetic acid:$ddH_2O$). Activity of MMPs could be observed as clear bands on a dark background.

Reverse Zymography

Briefly, 15% SDS polyacrylamide gels with gelatin (2.5mg/ml) and crude MMP-2(rhMMP-2, R&D Systems, USA) were prepared. Samples were loaded with a non reducing loading buffer and electrophoresed. Gels were then processed further in the same way as described for reverse zymography. After destaining, TIMP activities were detected as blue bands on a clear background. A regular SDS-PAGE gel for the samples was also run under identical conditions to discriminate between the TIMP and the protein bands, which also get stained by Coomassie blue.

**TUNEL (Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labelling) Evaluation of Apoptosis**

DNA fragmentation is a characteristic hallmark of apoptosis. TUNEL assay relies on the presence of nicks in the DNA which can be identified by Terminal deoxynucleotidyl

transferase (TdT), an enzyme that catalyzes the addition of dUTPs to the free 3'OH of cleaved DNA. The dUTPs are secondarily labelled with a marker (Gorczyka *et al*., 1992).

A TUNEL kit (Gen Script, USA) was used to detect the apoptotic cells in the regenerates. Briefly, tissues were excised using induced autotomy, frozen at -20ºC and embedded in OCT. Longitudinal cryosections (8-10µm) were taken on clean glass slides and fixed with cold acetone for 1-2 minutes. Sections were incubated with 0.1% Triton-X100 in PBS for 5-10 minutes, rinsed in PBS (2x5min), blocked using 3% $H_2O_2$ in methanol for 10 minutes at 25ºC and again rinsed with PBS twice. This was followed by incubation with TUNEL reaction mixture (Equilibration buffer, biotin-dUTP and TdT) for 45-60 minutes at 37ºC. Slides were rinsed thrice for five minutes each and then sections were kept in Streptavidin-HRP solution for 30 minutes at 37ºC. Sections were again rinsed, and DAB-$H_2O_2$ was added and kept for 5-10 minutes at 25ºC. After washing, sections were mounted with PBS:glycerol (1:1) and analyzed with a Leica DM2500 microscope. Apoptotic nuclei were stained dark brown. Same process was done for negative control sections, except that the TUNEL reaction mixture was devoid of TdT.

***In vivo* BrdU incorporation and Immunofluorescent localization**

A method commonly used to label cells in the S-phase of the cell cycle involves incorporation of the thymidine analog bromodeoxyuridine (BrdU) into replicating DNA and the subsequent immunohistochemical detection of the BrdU (Gratzner, 1982; Miller and Nowakowski, 1988). Administration of BrdU to living animals or addition of BrdU to culture medium can be used to determine relative proliferation rates, the length of the cell cycle, and the percentage of cells in the cell cycle (growth fraction) (Tang *et al*., 2007).

Intraperitoneal injection of BrdU (Sigma Aldrich, USA) at a dose of 100mg/kg body weight was given at different stages of regeneration and the regenerate was harvested by inducing autotomy after completion of one cell cycle. Tissues were embedded in OCT and fresh frozen sections (8-10µm) were taken on 0.01% poly-l-lysine coated slides. The sections were fixed in cold acetone (15-20 minutes at -20ºC) and air dried for 15 minutes followed by treatment with 2N HCl for 30-60 minutes at 37$^{o}$C. Sections were then rinsed in 0.1M borate buffer (pH 8.5) for 10 minutes (2x5 min) and then rehydrated in PBS at RT. Sections were blocked using normal serum (10% in PBS-BSA) for 1-2 hours at RT, and incubated with primary antibody [1:100 dilution of Mouse Anti-BrdU (Sigma-Aldrich, USA) in PBS] overnight inside a moist chamber at 4ºC. Next day, sections were washed with PBS (3x5min) and incubated with FITC conjugated secondary antibody [1:50 dilution of Goat Anti-Mouse IgG-FITC (Genei, Merck, USA) in PBS] for 2 hours at RT, washed, mounted with PBS:glycerol (1:1) and observed under a fluorescent microscope (Leica DM2500 utilizing LAS EZ software).

**Acridine Orange (AO) Staining of Nucleic acids**

Acridine orange (AO) is a cell permeant, nucleic acid selective, metachromatic fluorochrome useful for cell cycle determination. It interacts with DNA and RNA by intercalation or electrostatic attractions respectively. When AO bonds with DNA and forms a complex, it has an emission maximum of 525nm (green) and when it binds with RNA, the emission maximum shifts to 650 nm and the emitted light is red (Darzynkiewicz, 1990).

Regenerates were excised using induced autotomy and immediately transferred to a cryostat microtome maintained at -20ºC. After embedding in OCT, longitudinal cryosections (8µm) of these were taken on clean glass slides. Sections were immediately fixed in ice-cold acetone for 1-2 minutes, kept in phosphate buffer (pH 6.0) for 2-3 minutes and stained with a 1:10 dilution of Acridine Orange (Sigma-Aldrich, USA) stock solution (0.1% in phosphate buffer, pH 6.0) for 1-2 minutes. The sections were then observed using a fluorescent microscope (Leica DM2500).

**Statistical Analysis**

Morphometric data were subjected to Shapiro-Wilk test to analyse normality of distribution followed by The Mann-Whitney U Test to compare differences between the groups. All analyses were carried out by using SPSS 12.0 for Windows (SPSS Inc, Chicago, IL). The values are expressed as mean ± SE or as mode with range in parenthesis. A 'p' value of 0.05 or less was considered statistically significant. Graphs were prepared using GraphPad Prism (version 3.0 for Windows, GraphPad Software, San Diego, California, USA).

# CHAPTER 1

## INVESTIGATING THE PRESENCE OF FIBROBLAST GROWTH FACTOR 2 (FGF2) DURING TAIL REGENERATION OF LIZARD *HEMIDACTYLUS FLAVIVIRIDIS* AND ELUCIDATING THE SIGNIFICANCE OF FGF2 SIGNALLING IN THE PROGRESS OF REGENERATION

### INTRODUCTION

For centuries, the phenomenon of epimorphic regeneration, a complete reformation of lost tissues and organs after the development of body plans and cellular differentiation, has been a mystery of life and has fascinated many biologists (Nakateni *et al*., 2007). In invertebrate species including the hydra, planarians and arthropods, the regeneration of lost tissues and parts is widely observed (Slack, 2003), whereas most vertebrate species do not have such a remarkable ability for regeneration. Mammals only retain limited regeneration ability in the adult liver and infant fingertips (Hata *et al*., 2007; Yoshizato, 2007). However, among vertebrates, fish, urodeles and lizards have high regeneration abilities and express epimorphic regeneration. Epimorphic regeneration involves generation of new stem cells, either by proliferation of the existing stem cells or by dedifferentiation of adult cells, which differentiate to form the lost appendage that is more or less similar in size and structure compared to the original lost structure (Brockes and Kumar, 2002; Bryant *et al*., 2002).

Substantial research is being carried out in several animal models (*Ambystoma mexicanum*, *Notophthalmus viridescens*, *Xenopus laevis*) to understand the molecular mechanisms underlying epimorphic regeneration with an important objective to contribute to the knowledge of the expanding field of regenerative medicine and hoping to create useful therapies that could be applied to human injuries and diseases. As a result the molecules and cellular processes that play important roles in events like wound healing, inflammation, matrix reorganization, apoptosis, proliferation, differentiation and tissue patterning are now much better recognized. All these events are important processes of regeneration. Majority of such studies have been done to understand mechanisms of amphibian epimorphosis and the same is now vividly understood (Mullen *et al*., 1996; Ferretti *et al*., 2001; Stoick-Cooper *et al*., 2007). However, tail regeneration following autotomy is well known in several lizard species and the process of regeneration is comparable between the lizards and amphibians (Iten and Bryant, 1976). Understanding the mechanisms underlying this reptilian epimorphosis is important, as results can be better extrapolated to the mammalian system since reptiles are evolutionarily closer to mammals. An important difference between regenerative ability of amphibians and reptiles from that of mammals is that the former show

a scar-free wound healing, whereas in mammals a permanent fibrous scar tissue is formed as a result of tissue remodelling which appears to inhibit regeneration (Ferguson and O'Kane, 2004). If molecules known to be involved in amphibian regeneration play similar roles during reptilian regeneration, then regenerative mechanisms can be believed to be evolutionarily conserved across these vertebrate classes.

The precise coordination of several events involved in such a complex process requires cross talk and signalling between many factors and differential regulation of several genes. Neurotrophic factors derived from the nerve tissue are one such regulatory factors of regeneration. Most cells in the regenerating blastema and the regenerating epidermis are contacted by nerve terminals (Alibardi and Miolo, 1990). The trophic stimulation from spinal cord and regenerating ependymal tube have been deemed necessary to stimulate and sustain tail regeneration indicative of the importance of neurotrophic factors derived from the nerve tissue in the process (Simpson, 1964; Whimster, 1978; Alibardi *et al*., 1988). Studies on amphibian limb regeneration led to believe that FGF2 or other members of the FGF family is the neurotrophic factor required for regeneration, or is a mimic of the endogenous neurotrophic factor operating in the limb (Mullen *et al*., 1996). In fact, the FGF2 mediated rescue of denervated regenerates is the first demonstration of regeneration rescue by any means other than by nerves themselves (Singer, 1978).

FGFs are small peptide growth factors with multiple biological functions which play significant roles in patterning, growth and differentiation (Szebenyi and Fallon, 1999). FGF2, a member of this family is known to play key roles in development, remodelling and disease states in almost every organ system. Involvement of FGF2 in several developmental as well as regenerative processes such as limb development, angiogenesis, wound healing, and repair is well established (Obara *et al*., 2003; Yokoyama, 2008).

Wound healing predominantly involves apoptosis of damaged cells, extracellular matrix remoulding by proteases leading to cellular migration for covering the wound surface and eventually cellular proliferation to heal the wound. Several *in vitro* as well as studies on mammalian system have shown that FGF2 plays a very crucial role during wound healing (Chen *et al*., 1992; Pierce *et al*., 1992; Slavin *et al*., 1992; Tsuboi *et al*., 1992; Albertson *et al*., 1993; Legrand *et al*., 1993; Phillips *et al*., 1993; Gibran *et al*., 1994). Local application of FGF2 to skin wounds accelerates both dermal as well as epidermal wound healing (McGee *et al*., 1988; Hebda *et al*., 1990; Tsuboi and Rifkin 1990). Further, exogenous FGF2 is known to accelerate wound healing in different animal models as well (Tsuboi *et al*., 1990; Nissen *et al*., 1996). Topical application of FGF2 directly to the wound site in rats, augments the endogenous supply of FGF2 causing the recruitment and division of cells required for granulation tissue and subsequent contraction of the wound (Kuhn *et al*., 2001). Moreover,

apoptosis regulation following FGF2 administration to an incisional wound, may lead effectively to granulation tissue formation and promote a scar-less repair process in rats (Akasaka *et al*., 2004). FGF2 has been detected at the wound site early in healing and its rapid appearance after injury suggests that pre-existing tissue FGF2 may be important in healing rather than that synthesized *de novo* by inflammatory macrophages (Yoshimura *et al*., 2001).

Further, angiogenesis is essential for successful wound healing and tissue repair and FGF2 is a known angiogenic factor. FGF2 stimulates endothelial cells to produce both MMPs and VEGF and increases VEGFR expression. It also stimulates endothelial cell migration, pericyte attraction and matrix deposition (Presta *et al*., 2005). FGF2 induced activation of ERK1/2 is required for endothelial cell migration and hence, vessel formation (Pintucci *et al*., 2002). FGF2 can induce *in vitro* tissue factor (TF), which initiate thrombogenesis and FGF2 upregulation is involved in promoting vascular smooth muscle cell growth (Parenti *et al*., 2001). *In vivo*, the growth factors VEGF and FGF2 show a combined effect on both angiogenesis and maturation of blood vessels (Asahara *et al*., 1995). During corneal endothelial wound healing, endothelial migration is known to be induced by activated Cdc42 and inactivated Rho via PI3-kinase after FGF2 stimulation (Lee and Kay, 2006).

FGF2 is also known for its mitogenic potential and is actively involved in proliferation of various cell types. An increasing body of evidence shows that FGF2 produced by autosecretion or parasecretion promotes cell proliferation and inhibits apoptosis (Song *et al*., 2000; Sekimura *et al*., 2004). FGF2 promotes the proliferation of a wide range of mesoderm and neuroectoderm derived cells *in vitro* (Gospodarowicz *et al*., 1986; Folkman and Klagsbrun, 1987). Inappropriate expression of FGF2 and its receptors causes aberrant cell proliferation in various cancers and many human tumour cell lines (Chandler *et al*., 1999). Inhibition of either FGF2 or FGFR1 reduces ERK1/2 activation, cell proliferation and survival in uveal melanoma cells (Lefevre *et al*., 2009). Furthermore, FGF2 stimulates *in vitro* proliferation of blastema cells from regenerating limbs of newts (Albert *et al*., 1987).

Besides its involvement in several physiological and developmental processes, FGF2 is also known to be an important player of epimorphic regeneration, probably due to its involvement in events like wound healing, angiogenesis and cellular proliferation as described above. These processes are central to the event of regeneration as well. Hence, the fact that FGF2 has been implicated in successful regeneration in fish, amphibians and reptiles is not surprising (Mullen *et al*., 1996; Hata *et al*., 1998; Alibardi, 2010). FGF2 and its receptor is known to be present in several tissues during amphibian regeneration (Poulin *et al*., 1993; Ferretti *et al*., 2001) and more recently it is shown to be localized in regenerating tissues during tail regeneration of lizard *Lampropholis guichenoti* (Alibardi and Lovicu, 2010).

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). Lately, studies in our lab also revealed the role of FGF2 in another reptilian model the northern house gecko, *Hemidactylus flaviviridis*. FGF2 inhibition altered the regenerative process and caused a delay in attaining several regenerative stages (Yadav, 2005).

Hence, in view of the role of FGF2 in several physiological events as well as epimorphosis in diverse animal models, the current study was designed to establish the significance of FGF2 in tail regeneration of a reptilian model *H. flaviviridis.* The protein was localized in the tail regenerate of *H. flaviviridis* at different stages to learn about the tissues dependent upon this factor for their regeneration. Moreover, the levels of FGF2 were also quantified to get an idea of its changing levels and its requirement at different stages of regeneration. Furthermore, in order to elucidate its role in regulating progress of reptilian epimorphosis, the FGF2-FGFR1 signalling pathway was targeted using specific tyrosine kinase inhibitor SU5402 and the effects on successive stages of regeneration were observed. It is also important to evaluate the histological details underlying the regenerative process. Histological descriptions of the regenerative response have been previously illustrated for several lizard species (Bellairs and Bryant, 1985; Alibardi and Toni, 2005). However, such details for *H. flaviviridis* tail regenerate involving FGF2 signal inhibition have not been worked out and it becomes important to explore the structural alterations occurring in the tail regenerate due to the administration of FGFR1 inhibitor.

The present study examined whether this candidate neurotrophic factor of epimorphosis, functions in a similar manner during tail regeneration of *H. flaviviridis*, a reptilian model of regeneration which unlike fish and amphibian is an amniote that is evolutionarily higher in hierarchy and hence, might have evolved a different regulatory mechanism for epimorphosis. Further, lizard tail is projected as a potential model to study regeneration with a view to develop possible treatments for human diseases (Daniels *et al*., 2003). Moreover, understanding the regeneration mechanisms of lower vertebrate model systems is always aimed at achieving basic cues that could be useful in regenerative medicine.

## MATERIAL AND METHODS

### Experimental animals

Healthy adult Northern House Geckos, *Hemidactylus flaviviridis*, of both sexes with normal intact tail were collected and acclimated for a week before the commencement of the experiments. The animals were maintained in the animal house as per conditions described earlier (refer the section Material and Methods). All protocols of amputation and treatment were done under hypothermic anaesthesia (Reilly, 2001).

**EXPERIMENT I**

A total of 40 animals were used for this experiment. Autotomy was induced in these animals by exerting mild thumb pressure on the normal intact tail, two segments away from the vent. Animals which reached different stages of regeneration *viz*., wound epithelium (WE, appears as a smooth shining surface indicating healing of the wound), blastema (BL, approximately 2-4 mm in length from the stump) and differentiation (DF, 12-14 mm in length from the stump) stages on the same day were grouped and used for the studies. Another group of 5 lizards was used to collect intact tail samples for quantifying resting FGF2 level.

**FGF2 Quantification**

In this study, regenerates were collected from animals of each of the following stages: WE, BL and DF (14-15 mm in length from the stump) by inducing autotomy to release the regenerate along with the adjacent tail segment. The regenerates were then excised and processed for quantification. Samples of intact tail segment were also collected similarly (n=5). For protein extraction, samples were homogenized in 1:1 solution of PBS: protein lysis buffer and kept at ≤ -20° C for 1-2 hours. After two freeze thaw cycles, the homogenates were centrifuged at 9000RPM for 15-20 minutes at 4ºC. The supernatant was separated for protein estimation (BCA assay kit according to Smith *et al*., 1985). The samples were diluted to about 5µg protein/ml and quantified using an ELISA Kit (Quantikine, R&D Systems, for details see Materials and Methods). Colour intensity was read with an ELISA plate reader (Metertech ∑960) at a wavelength of 450 nm and concentration of FGF2 was computed from the standard readings.

**FGF2 Immunofluorescent Localization**

For this study the regenerating tail at defined stages of regeneration *viz*., WE, BL and DF were collected by inducing autotomy to release the regenerated tail along with an adjacent intact tail segment. These were embedded in Tissue-Tek OCT, frozen at -20$^{o}$C and further processed for immunolocalization.

Briefly, longitudinal cryosections (8-10µm) were fixed in acetone and then rehydrated with PBST followed by blocking with normal serum for 1-2 hours at room temperature (RT). Sections were then incubated with primary antibody (1:200 dilution of Rabbit Anti-FGF2) overnight inside a moist chamber at 4ºC. Following day, sections were washed with PBST and incubated with FITC conjugated secondary antibody (1:50 dilution of Goat Anti-Rabbit IgG-FITC) for 2 hours at RT. Sections were then washed with PBS and mounted in 1:1 mixture of PBS:glycerol and observed using a fluorescent microscope (Leica DM2500). Same procedure was followed for negative control sections except that these were incubated with antibody diluent instead of the primary antibody (for details see Material and Methods).

Reportedly, cross reactivity among species for FGF2 is high as it is a highly conserved protein among vertebrates. Hence, mammalian derived antibodies have been used in the present study considering this evolutionary conservation (Nugent and Iozzo, 2000)

**EXPERIMENT II**

**(A) Morphometric Analysis**

**1. Treatment before amputation**

A total of 18 animals were used and they were divided into two groups of nine animals each. Animals in each group were treated as follows:

Group 1: This group of animals served as a control to the experimental group and were injected with vehicle (1% DMSO).

Group 2: The animals of this group received SU5402 (0.7 mg/kg body weight).

A stock solution of SU5402 (Calbiochem®, EMD Biosciences, Inc., US) was prepared in DMSO and stored at $4^{o}$C. This stock was diluted to obtain a final dilution of SU5402 in 1% DMSO, which was used for treatment. Autotomy was induced in control and experimental animals as described earlier. The drug dosage was selected based on an initial dose range study. Drugs were administered *in loco* (at second intact tail segment from vent) at a maximum quantity of 0.075 ml/animal. Treatment started two days prior to amputation and was continued on every alternate day till the termination of the experiment (when control animals reached differentiation stage). The growth of the regenerate was measured at fixed intervals using a calibrated digital Caliper (Mitutoyo, Kawasaki, Japan) and time taken to reach defined stages of regeneration was recorded.

**2. Stage specific treatment**

Autotomy was induced, as described earlier, in 50 lizards, and the regenerating animals were selected at two defined stages of regeneration *viz*., (i) at completion of wound healing and appearance of wound epithelium (WE) stage, and (ii) at early blastema (BL) stage. Only those animals that attained the above stages on the same day were selected and grouped.

***(i) Treatment at WE stage***

Treatment commenced at WE stage and was continued on every alternate day till the animals were sacrificed. Eighteen animals that reached the WE stage on the same day were selected and divided into two groups of nine animals each and were treated as described earlier.

***(ii) Treatment at BL stage***

Eighteen lizards that attained the blastema stage on the same day were selected for the experiment. They were divided into two groups of nine animals each and treated as described above. Treatment started at the blastema stage and continued on alternate days as described earlier.

The time taken to reach the various stages of tail regeneration and the rate of growth of regenerate were recorded at fixed intervals. On successful completion of experiments the lizards were rehabilitated in standard laboratory diet and condition for two months and released back to their natural environment.

**Statistical Analysis**

The data were subjected to Shapiro-Wilk test to analyse normality of distribution followed by The Mann-Whitney U Test to compare differences between the groups. All analyses were carried out by using SPSS 12.0 for Windows (SPSS Inc, Chicago, IL). The values are expressed as mean ± SE or as mode and range in parenthesis. A 'p' value of 0.05 or less was considered statistically significant.

**(B) Histological study of tail regenerate of control and SU5402 treated *H. flaviviridis***

A total of 16 lizards of both sexes were selected and divided into two groups of 8 animals each.

Group I: This group of animals served as control to the experimental groups and injected with vehicle (1%DMSO).

Group II: The animals were injected with SU5402 (0.7 mg/kg body weight).

The procedures for autotomy and treatment remained same as described earlier with treatment starting prior to amputation. Control animals reaching the different stages of regeneration were selected and the regenerates along with an intact tail segment were collected by inducing autotomy. Regenerates of treated animals were collected on the day when the control animals reached the appropriate stages irrespective of whether treated animals attained the same stage or not. The regenerate was processed for histological analysis (see Material and Methods).

In short, Samples were fixed and decalcified. Paraffin wax blocks of the tissue samples were prepared. Longitudinal sections (7µm) of tails from three animals per regeneration group were stained with Harris's haematoxylin and eosin. The histological structure of the tissues on the slide was visualized using Leica DM2500 Microscope and pictures captured using EC3 Camera (utilizing LAS EZ software).

**RESULTS**

**EXPERIMENT I**

FGF2 localization was prominent during the initial wound healing and blastemal proliferative stages. Localization was also seen during the later stages of differentiation. FGF2 was mainly localized to the wound epithelium of the regenerating tail in the initial stages. Slight localization could also be observed in the region of accumulating blastemal cells. The spinal cord of the intact tail region was positively labelled for FGF2. As the regeneration progressed

further, intense FGF2 labelling could be detected in the wound epidermis and the external most layer of the epidermis which eventually cornifies was also intensely labelled. Good FGF2 labelling of the dermis with the fibroblasts along this region could be seen. Average labelling of the blastema and blood vessels could still be observed during this stage (Figure 1.5a (A-E)). The regenerating muscle bundles beneath the dermis, the regenerating ependymal tube and supporting cartilage tissue surrounding it were also positively labelled and showed good amount of fluorescence during blastemal proliferation (Figure 1.5b (A-B)).

During late differentiation stages the epidermis still maintained intense immunolabelling, whereas the regenerating muscles and ependyma with its supporting cartilage tissue showed average to good fluorescence. Scarce labelling could be detected in the region of regenerating connective and adipose tissue that lies between the cartilage and muscles. The dermis was also faintly labelled. Reasonably good immunofluorescence could be observed for the regenerating nerves seen during late regeneration (Figure 1.5b (C-E)). The immunolabelling can be concluded to be specific as the negative control sections showed negligible amount of fluorescence only in the external most layer of the epidermis with labelling almost absent in remaining regions (Figures 1.5a(F) and 1.5b(F)).

Quantification of FGF2 was done in the regenerating tail at different stages and also in the non regenerating tail to analyse the resting level of the protein and any changes in its levels during regeneration. From the results it could be observed that the FGF2 levels definitely increase with autotomy as compared to its value in the non regenerating tail. The amount of FGF2 remained high till the blastemal proliferation continues with the maximal amount also observed during blastema stage. Its amount started to decline towards much later stages of differentiation but the results shown here account for the FGF2 level assayed during mid differentiation period and probably so were still recorded higher than the resting FGF2 level. FGF2 quantified during wound healing stage amounted to 80.34±0.688ng/g tissue, 96.98±2.06ng/g tissue during blastema and 75.2±1.802ng/g tissue during late regeneration. The resting level of FGF2 was approximated to 61.39±2.41ng/g tissue (Values are expressed as Mean ± S.E.) (Figure 1.1).

**EXPERIMENT II**

**Progression of regeneration in *H. flaviviridis* subjected to treatment before amputation**

In this experiment, SU5402 treatment started prior to amputation and it was observed that blocking FGF2 signalling significantly hampered the progression of epimorphosis. Rate of growth and percentage of growth inhibition were calculated for the growth (2-12 mm) and differentiation (12-24 mm) stages of regenerating tail. It was observed that SU5402 treatment significantly decreased the growth rate of regenerate during both 2-12 mm ($p \leq 0.01$) and 12-

24 mm stages of growth ($p \leq 0.05$). A significant decrease was observed in the length of the regenerate of all SU5402 treated animals with 59% reduction in growth rate during 2-12 mm stage and 27% reduction during 12-24 mm stage (Tables 1.1a, b; Figure 1.2).

**Progression of regeneration in *H. flaviviridis* subjected to stage-specific treatment**

To understand the role played by FGF2 at different stages of tail regeneration, lizards were treated at distinct stages of regeneration *viz*., WE and BL.

***(i) Treatment at WE stage***

Injection with FGF2 receptor inhibitor at the wound epithelium stage delayed attainment of subsequent stages of regeneration. Lizards treated with SU5402 took an additional 5 days to attain the blastema stage as compared to control animals. Moreover, the treatment group reached the differentiation stage on an average of 23 days, whereas control animals took only 13 days to attain the same. Further, a definite decrease ($p \leq 0.01$) in the rate of growth of regenerate was observed in SU5402 treated animals with a mean reduction of 46% during 2-12 mm stage and 17% during 12-24 mm stage of regeneration (Tables 1.2 a, b; Figure 1.3).

***(ii) Treatment at BL stage***

In contrast to the first two experiments, treatment with SU5402 at the blastema stage showed only marginal influence on the progression of regeneration. A delay of two days in attaining the differentiation stage was observed in the treatment group. However, even though the progression of growth was hampered during initial stages of growth, no statistically significant reduction in rate of growth was recorded during 12-24 mm stage (Tables 1.3a, b; Figure 1.4).

**Histological observations in tail of control and SU5402 treated *H. flaviviridis***

***(a) At Wound epithelium (WE) stage***

Control tail sections at this stage showed defined structures like a well formed WE, which was a few layers thick at the apex, aptly called the apical epithelial cap (AEC). The regenerating ependymal tube, several blood vessels and connective tissue were also evident. Further, cells accumulating towards the wound epithelium could also be seen, which will eventually form the blastemal cone (Figure 1.6 D). However, the wound surface of tail sections from treated animals showed traces of blood clots indicating that the wound is yet to heal. No distinct ependymal tube was present in the tail sections of treated lizards. Blood vessels nourishing the regenerate were also found significantly reduced. Ultimately all these affected structures are important determinants necessary for eventual proliferation. Thus, accumulation of cells leading to blastema too was not prominently seen in the treated caudal sections (Figure 1.7D).

***(b) At Blastema stage***

Control sections at blastema stage showed a thicker, multilayered WE and a thin layer of dermis beneath. The ependymal tube was properly evident. An additional feature noted during this stage was the appearance of procartilagenous tissue accrued surrounding the ependyma. Regenerating muscle bundles, seen as separate aggregates beneath the dermis, were another prominent histological feature seen during this stage. Several blood vessels and a pool of proliferating blastemal cells immediately beneath the WE could also be seen (Figure 1.6E). In the treatment group, by this time, initial signs of wound healing began to appear. A thin WE was formed, with few blood vessels seen in the region beneath it. A small area of proliferating cells immediately following a faintly regenerated ependymal tube was also evident. However, cartilage and muscle tissue were still not properly manifested (Figure 1.7E).

***(c) At Differentiation stage***

This stage accounts for the complete re-growth of the lost tail with proper tissue architecture. A well developed epithelium including both the epidermis and dermis was observed. Pronounced ependymal growth could be seen with well developed cartilaginous tissue surrounding it. Properly regenerated muscle bundles with well formed connective tissue and adipose tissue were observed (Figure 1.6F). With the formation of muscle bundles, the regenerate from the treatment group at this stage showed signs of mesenchyme differentiation. An elongating ependyma finally became evident with traces of regenerating cartilage surrounding it. The zone of proliferating blastemal cells was also seen evidently beneath a proper epithelium (Figure 1.7F). However, the tissue architecture observed in the SU5402 treated tail at differentiation stage was poorly organised compared to that of control at the same stage and was equivalent only to a tail at the blastema stage, indicating a definite delay in regeneration.

A dorsal view of the gross morphology of the regenerating tail during different stages has also been shown in figures 1.6A-C and 1.7A-C indicating the delayed regeneration in treated animals.

Thus, it is evident from the current observations that the inhibition of FGF2 signalling hampers the reptilian tail regeneration. However, once the regenerate reach the initial phase of growth, further progression is independent of FGF2 signalling as could be concluded from the stage specific experiments. Therefore, it is likely that FGF2 is one of the key molecules that direct the early events of the reptilian regenerative process nonetheless, its involvement, if at all, during the later stages of repatterning and differentiation is trifling. These morphometric observations were corroborated by histological studies. Further, it is apparent from the histological profile that the ablation of FGF2 signalling affects formation of a proper wound epithelium that in turn might affect several important signals emanating from it.

Consequently, subsequent proliferation and differentiation of several tissues might get affected.

## DISCUSSION

Nerve dependence is a common feature of appendage regeneration in many contexts and may be a mechanism to ensure that the regenerate is functionally innervated (Brockes and Kumar, 2008). Regeneration of the salamander limb is absolutely dependent on concomitant nerve regeneration and is abrogated by denervation (Singer, 1952). The events of wound healing and generation of blastemal cells are both apparently unaffected by prior denervation, but the subsequent proliferation of the blastemal cells is severely curtailed (Maden, 1978; Kintner and Brockes, 1985; Goldhamer *et al*., 1992). The regenerating axons and the wound epithelium are key accessory "niche" tissues for the blastemal cells, and the interplay between these two tissues is a critical feature of regeneration. Studies on amphibian tail and limb regeneration indicated that candidate neurotrophic factors involved in this process include the prototypic FGFs -the acidic and basic fibroblast growth factors (FGF1and FGF2), (Brockes, 1984; Mescher, 1996; Geraudie and Ferretti, 1998). Since FGF1 lacks the ability to rescue the growth of a denervated blastema, it is not considered as a true neurotrophic factor (Dungan *et al*., 2002). Instead, FGF2 can rescue a denervated blastema (Mullen *et al*., 1996) and is thought to stimulate blastemal cells to produce FGF1 that activates the apical cap to further produce FGF1 and FGF2 that feed-back to blastema cells and maintain their proliferative state (Alibardi and Lovicu, 2010).

In the current study, FGF2, the established neurotrophic factor of amphibian regeneration was localized in the regenerating tail of lizard, *H. flaviviridis* with a view to signify its presence as necessary for reptilian regeneration as well. The wound epithelium is one of the target tissues labelled intensely for this protein throughout the entire study. Blastemal cells are also labelled though not intensely. It appears as if the action of FGF2 on wound epithelium is necessary to trigger the trophic stimulation that eventually allows blastemal cell accumulation and proliferation. Blood vessels are also labelled positively. FGF2 has many biological activities that stimulate the proliferation of fibroblast and capillary endothelial cells, thus promoting angiogenesis and wound repair (Abraham *et al*., 1986; Montesano *et al*., 1986; McGee *et al*., 1988; Gospodarowicz, 1990). FGFs have been reported to be distributed in the wound epidermis, and in particular in the apical cap of the regenerating limb of the newt, and it may stimulate the blastemal cells to induce cell replication factors (Dungan *et al*., 2002; Giampoli *et al*., 2003). An increased production of FGFs and their receptors in the wound epidermis has also been reported for mammalian skin and hairs (DuCros *et al*., 1993; Takenaka *et al*., 2002). FGF2 is present in both apical epithelial cap (AEC) and in nerves and its levels decrease in response to denervation suggesting that there is a link between innervations and the formation of an AEC that is able to sustain distal outgrowth (Mullen *et al*., 1996). FGF2

also has a critical role for blastema proliferation and maintenance. The FGF2 receptor FGFR1 is distributed throughout blastemal mesenchyme during newt limb regeneration, suggesting that FGF2 could promote mitotic activity of blastemal cells (Poulin *et al*., 1993). It also promotes blastemal growth during zebrafish fin regeneration (Hata *et al*., 1998)

Differentiating tissues in the regenerating *H. flaviviridis* tail *viz*., the regenerating muscles, ependyma, nerves and cartilage also labelled positively for FGF2 indicating that formation and proliferation of these tissues is dependent upon this factor. Weak labelling of connective and adipose tissues indicated a lesser role of this protein in their differentiation. FGFs have been reported to be distributed in the differentiating and growing muscle bundles of the regenerating lizard tail (Bellairs and Bryant, 1985; Alibardi, 1995). FGF2 and FGF1 are also present in developing and regenerating muscles of mammals, where these growth factors may have autocrine stimulation for their differentiation and growth (Joseph-Silver stein *et al*., 1989; Anderson *et al*., 1991).

FGF2 expression is induced in the regenerating spinal cord in amphibians only after tail amputation, and is expressed in the undifferentiated cells lining the ependymal canal from which new cord will form (Zhang *et al*., 2000). Also neural precursors isolated from adult rat brain are induced to proliferate and to differentiate by FGF2 (Richards *et al*., 1992). FGF2 is also involved in mammalian nerve regeneration and it is up-regulated after peripheral nerve crush (Grothe and Nikkah, 2001). While FGF2 acts in vitro on both astroglial cells and neurons, the mature oligodendrocytes are induced to dedifferentiate and to proliferate by FGF2 supporting a mechanism for regeneration of the oligodendroglial lineage after demyelination (Grinspan *et al*., 1993). FGF2 is also well known as a potent regulator of functions of bone and cartilage cells. It is produced by cells of osteoblastic lineage, accumulated in bone matrix and acts as an autocrine/paracrine factor for bone cells (Canalis *et al*., 1988; Rodan *et al*., 1989, Hurley *et al*., 1994). Proliferation and differentiation of osteoblasts is also stimulated by FGF2 (Globus *et al*., 1988). Overall, FGF2 is known to be distributed in the regenerating spinal cord, subset of blastemal cells, basal epidermal layer, differentiating muscles and chondroblasts (Ferretti *et al*., 2001) during amphibian limb regeneration and a similar pattern of distribution is also observed for the reptile under study. Conclusively, the mechanism by which FGF2 influences epimorphic regeneration can be said to evolutionarily conserved among two vertebrate classes-amphibians and reptiles.

FGF2 protein levels were also measured at different stages of regeneration. High levels of this protein were recorded after autotomy with the highest amount at the blastemal stage Normally FGF2 does not have a signal sequence for cell secretion through golgi apparatus (Saksela and Rifkin., 1990; Friesel and Maciag, 1995; Dahl *et al*., 2000) and it is probably released extracellularly only after cell damage. According to this hypothesis, it is speculated

that FGF2 plays a negligible role in normal stage, but with increasing damage more FGF2 is released and stimulates neurogenesis (Yoshimura *et al*., 2001). The injury to blood vessels and nerves, which occurs as a result of amputation, is thought to be a trigger for the release of FGF2 (Zhang *et al*., 2000; Yoshimura *et al*., 2001). Once this preformed FGF2 is released, it further activates the synthesis and release of more FGF2 in an autocrine manner (Yoshimura *et al*., 2001)

A subsequent decrease in FGF2 was observed with progressive regeneration and a low level was recorded in the regenerate at mid differentiation stage as compared to initial stages indicating towards the pattern of temporal requirement of this protein during regeneration. It has been reported that FGF2 inhibits skeletal muscle differentiation in chick (Kruzhkova and Burgess, 2000). Recently it has been shown that HSPG glypican-1 acts as a positive regulator of muscle differentiation by sequestering FGF2 in lipid rafts and preventing its binding and dependent signalling (Gutierrez and Brandan, 2010). Moreover, analysis of MM14 mouse myoblasts demonstrated that terminal differentiation is repressed by pure preparation of FGF2 (Clegg *et al.,* 1987) and FGF repression occurs only during the G1 phase of the cell cycle by a mechanism that appears to be independent of ongoing cell proliferation. Further, in denervated late limbs of larval *X. laevis*, FGF2 expression at the level of amputation surface of late limbs is related to wound healing, considering that FGF2 plays a role in angiogenesis and tissue repair, but is not sufficient to promote blastema formation in the absence of FGF2 released from nerves (Cannata *et al*., 2001). Nevertheless, whether FGF2 has an inhibitory action during differentiation or the differentiated state cells are not capable of secreting sufficient amount of FGF2, both the facts indicate a lesser role of FGF2 during late differentiation and patterning.

Evidently, the current study establishes FGF2, the crucial neurotrophic factor of amphibian regeneration as a candidate neurotrophic factor of tail regeneration in a reptilian model *H. flaviviridis* as well. Hence, FGF2 inhibition studies to further analyze the mechanisms by which FGF2 regulates reptilian regeneration were explored. FGF2 signalling was inhibited by using FGFR1 inhibitor SU5402 and effects on progression of regeneration were studied.
That FGF2 is one of the main neurotrophic factors affecting epimorphic regeneration in amphibian and fish model systems is a well known fact (Mullen *et al*., 1996; Hata *et al*., 1998) morphometric observations amply testified that FGF2 signalling was imperative for regenerative response in lizard *H. flaviviridis* as well. Inhibition of FGF2 signal by treatment with SU5402 hampered the wound healing process and delayed the formation of a proper wound epithelium during tail regeneration. Formation of a functional wound epithelium is essential for successful regeneration as it provides the necessary signals for the underneath tissues to dedifferentiate, proliferate and to form the blastema (Lo *et al*., 1993; Kumar *et al*., 2000). Impaired wound healing in the SU5402 treated lizards must be due to inadequate

FGF2 signalling. FGF2 is a potent mitogen and was involved in epithelial cell proliferation and migration taking place during wound healing. It has also been shown that FGF2 incorporated chitosan hydrogel may be a promising wound dressing, especially in the treatment of healing impaired wounds (Obara *et al*., 2003). Besides, the process of wound healing is known to be controlled by critical events like re-epithelization, angiogenesis and matrix deposition (Miller and Gay, 1992), and it has been hypothesized that FGF2 might be involved in these processes.

Moreover, wound healing is closely complemented by cell accumulation, leading to the appearance of the blastemal cone. Blastema formation is a major cue in epimorphic regeneration. It has been postulated that the basis for regenerative competence resides in the capability of an adult organism to induce a local and discrete population to re-enter the cell cycle in a highly controlled manner (Tsonis, 1996; Brockes, 1997; Stocum, 1999). The proliferative blastemal cells can then eventually give rise to all of the cell types necessary for the complete regeneration of the lost structure (Clause and Capaldi, 2006). FGF2 is reported to be the endogenous mitogenic factor responsible for blastema formation and growth in amputated and denervated early limbs of *X. laevis* (Cannata *et al*., 2001). It regulates blastemal proliferation during fin regeneration as well (Poss *et al*., 2000; Tawk *et al*., 2002). In the current study also, SU5402 treatment, both before amputation and at WE stage, delayed this initial growth and blastema formation in *H. flaviviridis*. Evidently, the pool of accumulating blastemal cells and their proliferation leading to formation of blastemal cone was affected by impaired FGF2 signalling. Further, the formation of blastema involves extensive remodelling of the extracellular matrix, for which activity of matrix metalloproteinases (MMPs) is required (refer chapter 2). Since, FGF2 is known to increase the activity of MMPs (Palmon *et al*., 2000), it is possible that the process of matrix reorganization in the animals treated with SU5402 might have been affected which could be an additional reason for delayed formation of blastema in treated lizards.

Further, once the blastema is formed, the cells get engaged in repeated cycles of cell division leading to the increase in the length of the regenerate. Injection of SU5402 before autotomy and at WE stage, curtailed the rate of growth of regenerate. This decrease in the growth rate of regenerate was more significant from 2-12 mm. Growth rate of regenerate from 12-24mm was also decreased in the treated lizards, but it was not significant statistically. This prompted one to propose that once the regenerate accomplish a certain length and commence differentiation it no more requires FGF2 signalling for the furtherance of its growth. This notion gains credence from the current stage specific treatment wherein it was observed that the growth rate of regenerate in animals that received SU5402 at blastema stage was not significantly different from that of the control animals. As discussed earlier, FGF2 is known to inhibit or at least have a lesser role during differentiation of muscle tissue (Clegg *et al.*,

1987; Kruzhkova and Burgess, 2000; Gutierrez and Brandan, 2010). Also *in vitro*, FGF2 is known to block oligodendrocyte maturation/differentiation (Goddard *et al*., 1998). However, differentiation of certain cell types such as fibroblasts, neuroectodermal cells and melanocytes is also reportedly dependent on FGF2 (Halaban *et al*., 1992; Bhora *et al*., 1995; Gibran *et al*., 1994).

In order to complement the morphometric observations and to identify the target tissues affected by impaired FGF2 signalling, histological profile of the tail sections was also analysed in the present study. The observations revealed that the several events of epimorphosis like formation of the wound epithelium, recruitment and proliferation of blastemal cells, differentiation of mesenchymal cells such as muscle bundles, ependymal growth and regeneration of the supporting cartilage surrounding the ependyma are delayed in the SU5402 treated lizards. Blood vessel formation is also evidently being downplayed in the treatment group. These results are indicative of the importance of FGF2 signalling in the timely restoration of a lost appendage with proper tissue integrity even in a reptilian model. A careful scan through the archived reports (Gospodarowicz *et al*., 1986; Nissen *et al*., 1996; Sekimura *et al*., 2004; Presta *et al*., 2005) revealed that FGF2 indeed play a definite role in many of the developmental events reported in the current study. Alibardi, (1999 and 2001) while studying limb regeneration in newts noted that the wound epidermis start as an immature keratin layer and the formation of the granulated layer occurs by the accumulation of keratohyalin-like granules. FGF2 has been reported to induce differentiation of keratinocytes (Werner *et al*., 1993). Moreover, FGF2 has been localized to the apical cap during newt limb regeneration and is reported stimulating blastemal cells to respond to cell replication factors (Giampoli *et al*., 2003).

Further, FGF2 has been detected in the basal lamina of the blood capillaries, primarily at sites of vessel branching, and in the endothelium of the capillaries of some tumours (Dimario *et al*., 1989; Cordon-Cardo *et al*., 1990) suggesting that endothelial cell derived FGF2 may mediate angiogenesis with an autocrine mode of action. Angiogenesis is tightly regulated by several extracellular signals with one of the most relevant agents being FGF2 (Bikfalvi *et al*., 1997) that binds to tyrosine kinase receptors (RTKs) and activates intracellular signalling cascades partially overlapping with those initiated by other factors, such as VEGF (Cross and Claesson-Welsh, 2001).

There are many reports regarding the involvement of FGF2 in the regeneration of nerves during epimorphosis. It plays a role both in the early stages of regeneration, possibly in the proliferation of neural progenitors and in the maintenance of the undifferentiated state (Ferretti *et al*., 2001). It has been reported that after spinal lesion in salamander, FGF2 may be involved in functional recovery of locomotion by influencing cell proliferation and /or

neuronal differentiation (Moftah *et al*., 2008). Moreover, FGF2 administration following spinal cord injury promotes hindlimb movement recovery in the adult rat (Rabchevsky *et al*., 2000). Exogenous application of FGF2 has stimulatory effects on bone formation in several *in vivo* models as pharmacological action (Aspenberg and Lohmander, 1989; Kawaguchi *et al*., 1994, Kawaguchi *et al*., 2001). FGF2 deficient mice even exhibit decreased bone mass and bone formation; although changes are rather moderate (Montero *et al*., 2000). Stimulatory effects of FGF2 on cartilage formation have also been noted in several animal models (Aspenberg and Lohmander, 1989; Kawaguchi *et al*., 1994; Nakamura *et al*., 1997).

Thus, it is apparent that FGF2 more or less orchestrate a plethora of developmental events *viz.,* formation of wound epithelium, development of blood vessels, nerve regrowth, myogenesis and chondrogenesis in various animal models during post embryonic development. These events are also quintessential milestones of epimorphic regeneration and hence, FGF2 signalling is indispensable for normal regenerative response. Furthermore, the results of the current study proved beyond doubt that the FGF2 signalling plays not only a pivotal role in anamniote appendage regeneration but is also vital for epimorphic regeneration in amniote which in another term is an ample testimony to the evolutionarily conserved mechanisms of developmental regulation.

A successful wound healing response inevitably requires extensive rearrangement of cells which is possible only after regulated proteolysis of the extracellular matrix. Since FGF2 is now undoubtedly known to influence wound healing in *H. flaviviridis* tail regeneration, it might be involved in this matrix reorganization. Moreover, regeneration also involves upregulation and/or downregulation of several proteins and it is important to know whether FGF2 as an important regulator of reptilian epimorphosis has a role in this process of protein turnover. Hence, effects of inhibited FGF2 signalling on proteolytic activities as well as protein turnover during regeneration were studied further.

**Table 1.1a: Number of days taken to reach various regenerative stages in *H. flaviviridis*, subjected to *in loco* injection of SU5402 before amputation.**

| Treatment | No. of Days | | |
|---|---|---|---|
| | **WH** | **BL (2mm)** | **DF (12mm)** |
| Control | 5(4-5)# | 8 (8-9) | 13 (12-13) |
| SU5402 Treated | 10(10-11) | 15(15-16) | 26 (25-26) |

**Table 1.1b: Length of tail regenerated in *H. flaviviridis* after *in loco* treatment with SU5402 before amputation.**

| Treatment | Rate of Growth of Regenerate (mm/day) | | % decrease (↓)compared to control | |
|---|---|---|---|---|
| | **2-12mm** | **12-24mm** | **2-12mm** | **12-24mm** |
| Control | 2.3±0.122@ | 1.83±0.069 | - | - |
| SU5402 Treated | 0.946±0.022** | 1.33±0.099* | 59[$]↓ | 27↓ |

**Table 1.2a: Number of days taken to reach various regenerative stages in *H. flaviviridis*, subjected to *in loco* injection of SU5402 at WE stage.**

| Treatment | No. of Days | | |
|---|---|---|---|
| | **WH** | **BL (2mm)** | **DF (12mm)** |
| Control | 5(5-6)# | 9 (8-9) | 13 (13-14) |
| SU5402 Treated | 5(5-6) | 14(14-15) | 23 (22-23) |

#Values are expressed as mode and range in parenthesis

@Values are expressed as mean±SE, *n*=5, * p<0.05, **p<0.01

[$]Values are corrected to the nearest whole number

**Table 1.2b: Length of tail regenerated in *H. flaviviridis* after *in loco* treatment with SU5402 at WE stage.**

| Treatment | Rate of Growth of Regenerate (mm/day) | | % decrease (↓)compared to control | |
|---|---|---|---|---|
| | 2-12mm | 12-24mm | 2-12mm | 12-24mm |
| Control | 2.2±0.122@ | 1.77±0.057 | - | - |
| SU5402 Treated | 1.194±0.034** | 1.466±0.034** | 46[$]↓ | 17↓ |

**Table 1.3a: Number of days taken to reach various regenerative stages in *H. flaviviridis*, subjected to *in loco* injection of SU5402 at BL stage.**

| Treatment | No. of Days | | |
|---|---|---|---|
| | WH[a] | BL (2mm) | DF (12mm) |
| Control | 5(5-6)# | 9 (9-10) | 14 (13-14) |
| SU5402 Treated | 5(5-6) | 9(9-10) | 16 (15-16) |

**Table 1.3b: Length of tail regenerated in *H. flaviviridis* after *in loco* treatment with SU5402 at BL stage.**

| Treatment | Rate of Growth of Regenerate (mm/day) | | % decrease (↓)compared to control | |
|---|---|---|---|---|
| | 2-12mm | 12-24mm | 2-12mm | 12-24mm |
| Control | 2.2±0.122@ | 1.77±0.057 | - | - |
| SU5402 Treated | 1.522±0.056** | 1.6±0.099 | 31[$]↓ | 9↓ |

#Values are expressed as mode and range in parenthesis

@Values are expressed as mean±SE, n=5, **p<0.01

[$]Values are corrected to the nearest whole number

**Figure 1.1. FGF2 levels during different stages of tail regeneration as compared to resting FGF2 level in tail of *H. flaviviridis*.**

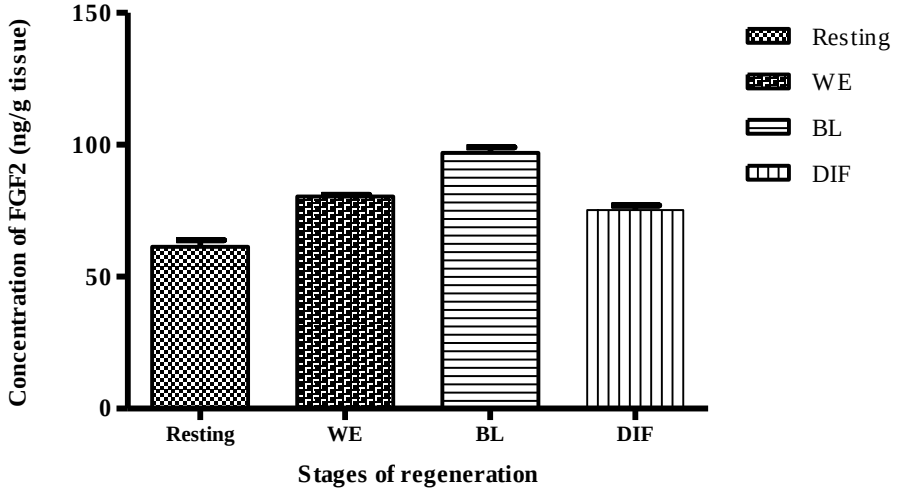



**Figure 1.2. Progression of tail regeneration in Control and SU5402 treated *H. flaviviridis* (Treatment before amputation)**

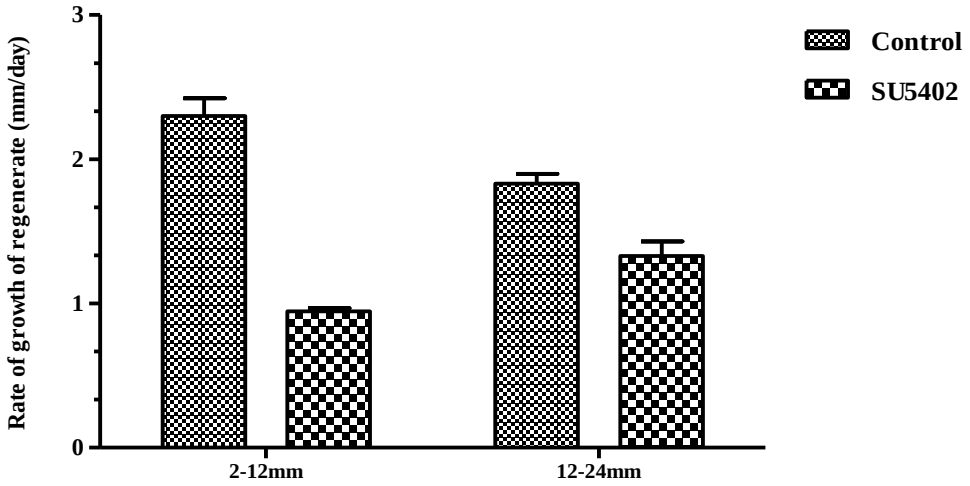


**Figure 1.3. Progression of tail regeneration in Control and SU5402 treated *H. flaviviridis* (Treatment at WE stage)**

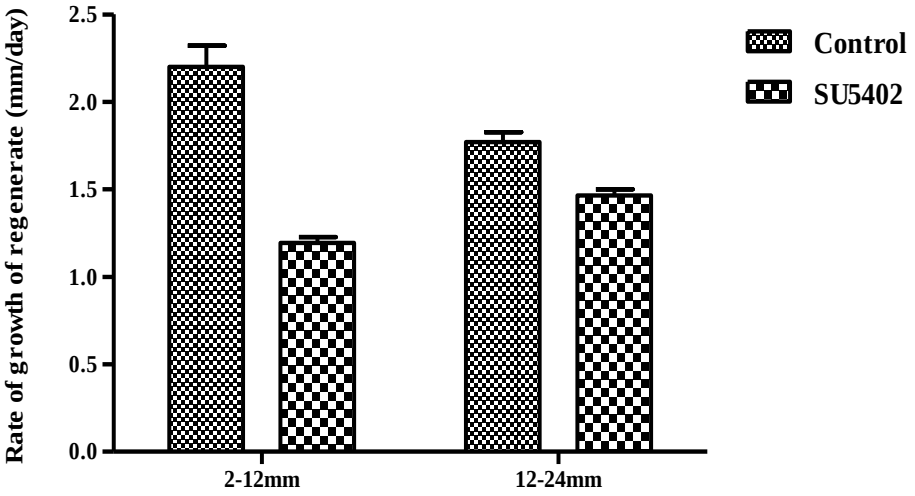



**Figure 1.4. Progression of tail regeneration in Control and SU5402 treated *H. flaviviridis* (Treatment at BL stage)**

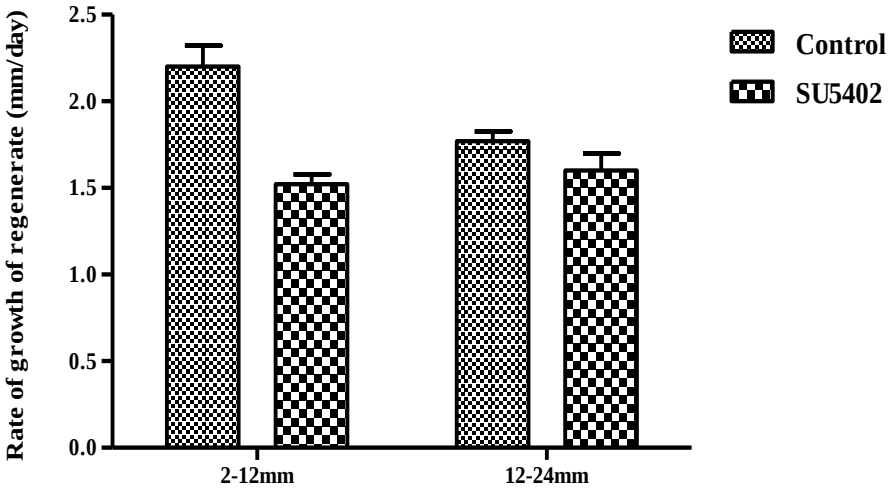
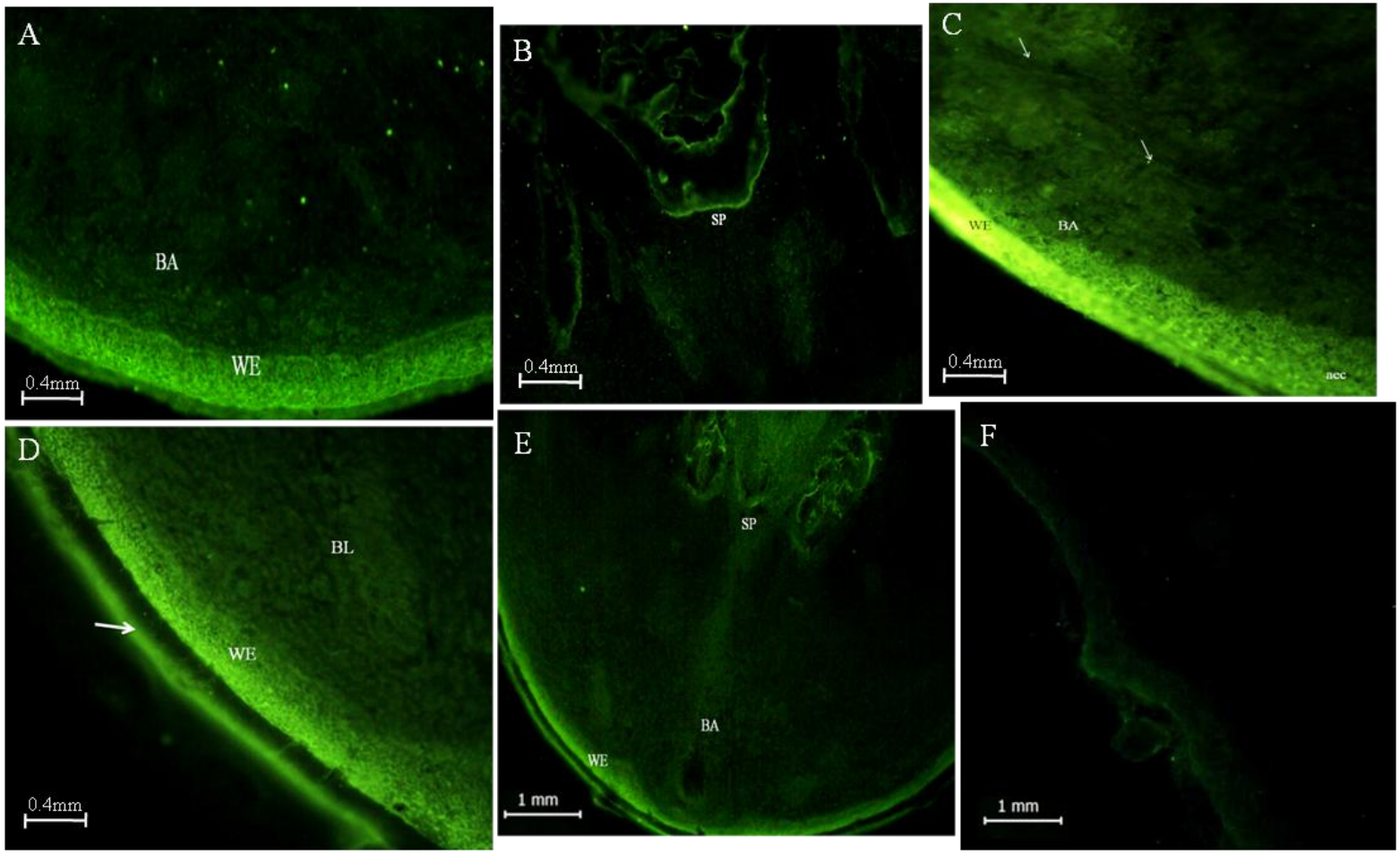
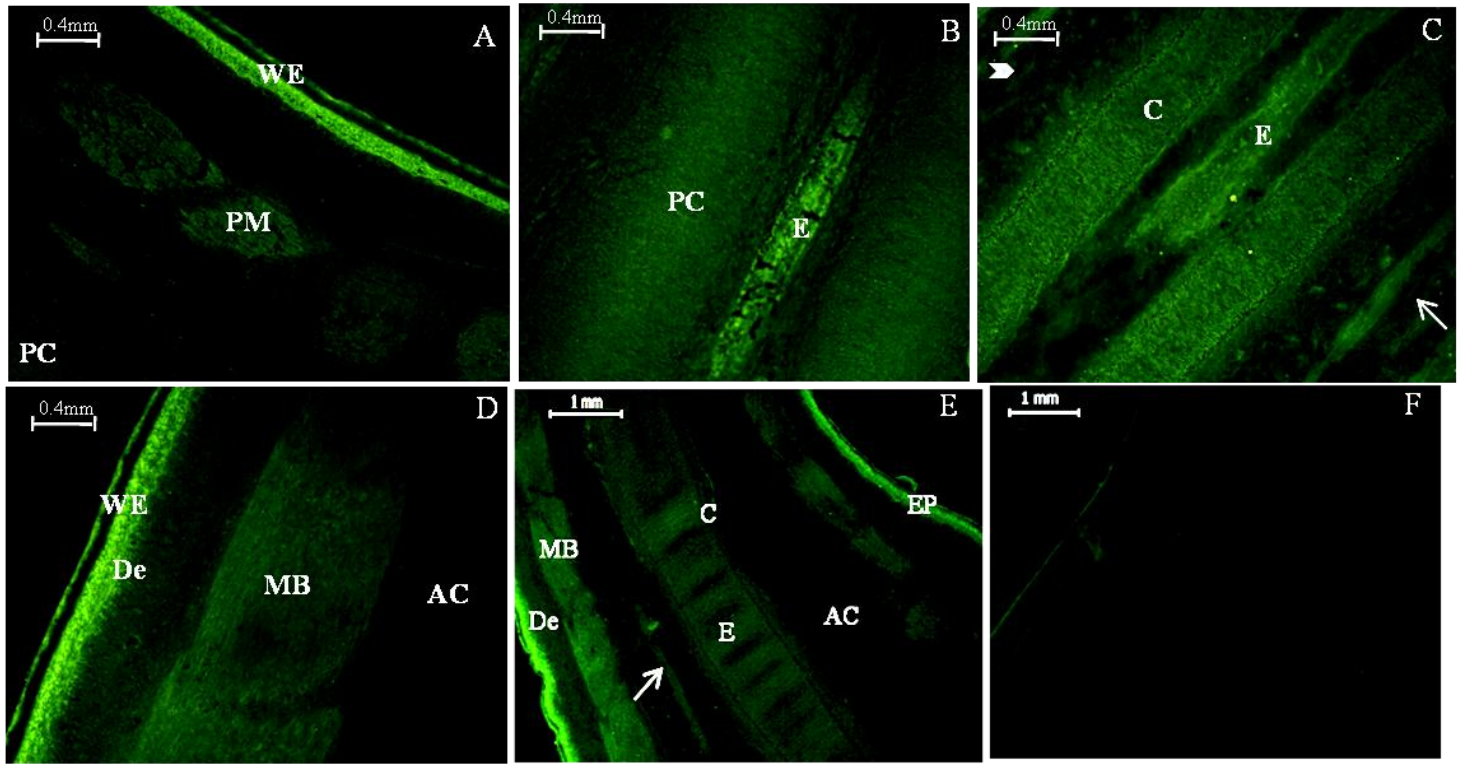


**Figure 1.5a Immunofluorescent localization of FGF2 in regenerating tail of *Hemidactylus flaviviridis* during different stages**




**(A)** Intense FGF2 labelling in wound epithelium with slight labelling of blastemal accumulation after wound healing **(B)** Spinal cord positively labelled during WE stage **(C)** Thick apical epithelial cap seen at the apex positively labelled for FGF2 **(D)** Blastema stage with outer cornaceous layer labelled in addition to the WE **(E)** Overall pattern of FGF2 distribution after formation of WE **(F)** Negative Control
aec- apical epithelial cap, BA- cell accumulation prior to blastema formation, BL- blastema, SP- spinal cord; arrows indicate blood vessels labelled for FGF2

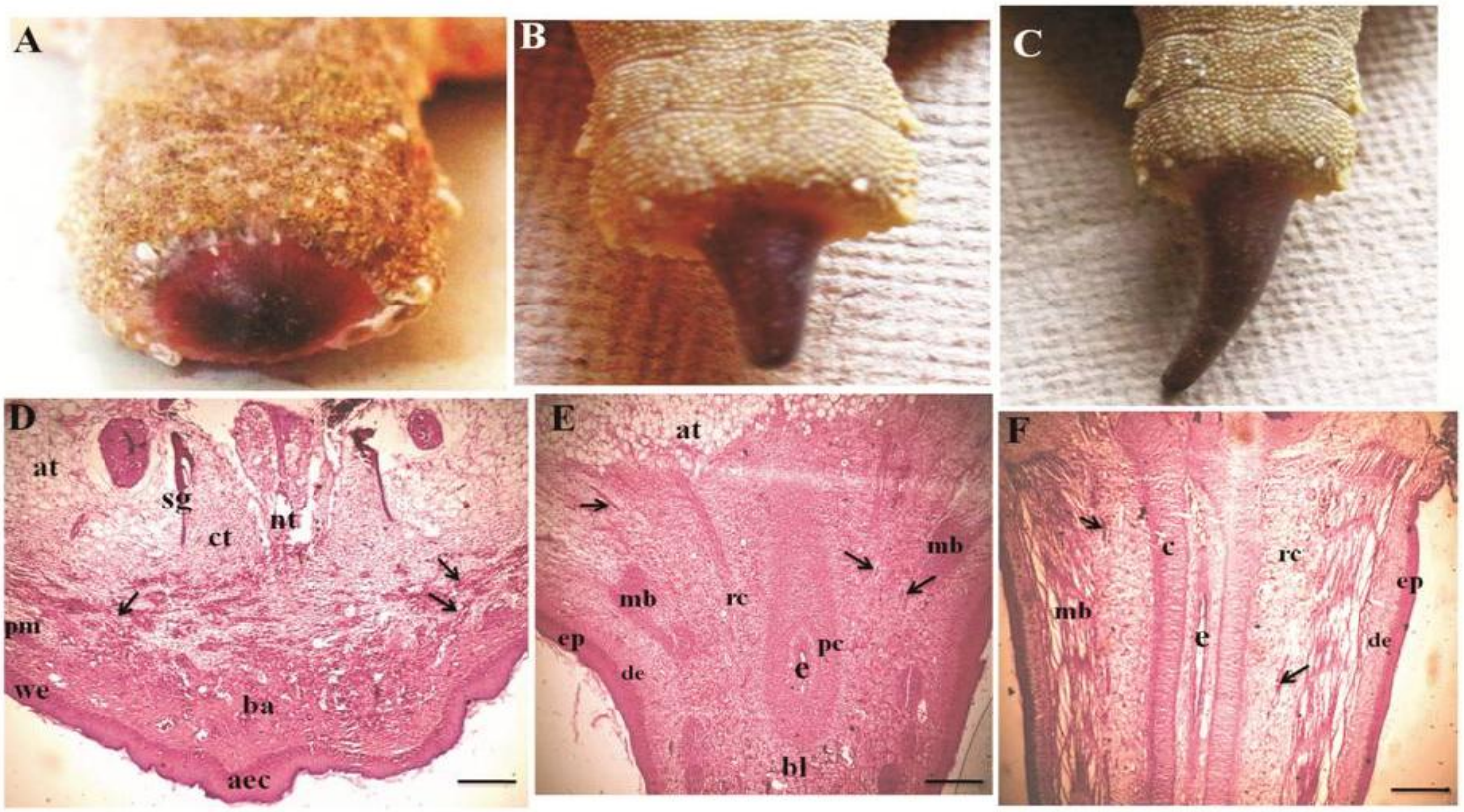
**Figure 1.5b Immunofluorescent localization of FGF2 in regenerating tail of *Hemidactylus flaviviridis* during different stages**




**(A)** Strong FGF2 labelling in the promuscle bundles during blastema stage **(B)** Procartilage and ependyma labelled positively during blastema stage **(C)** Differentiation stage labelling of ependyma and cartilage **(D)** Differentiation stage positive labelling of regenerated muscle **(E)** Overall view of FGF2 labelling during differentiation **(F)** Negative Control

AC- regenerated adipose and connective tissue, C- cartilage, D- dermis, E- ependyma, EP- epidermis, PC- procartilage, PM- promuscle, MB- muscle bundle, WE- wound epithelium; Arrows and arrowhead indicate regenerating nerves and blood vessels respectively

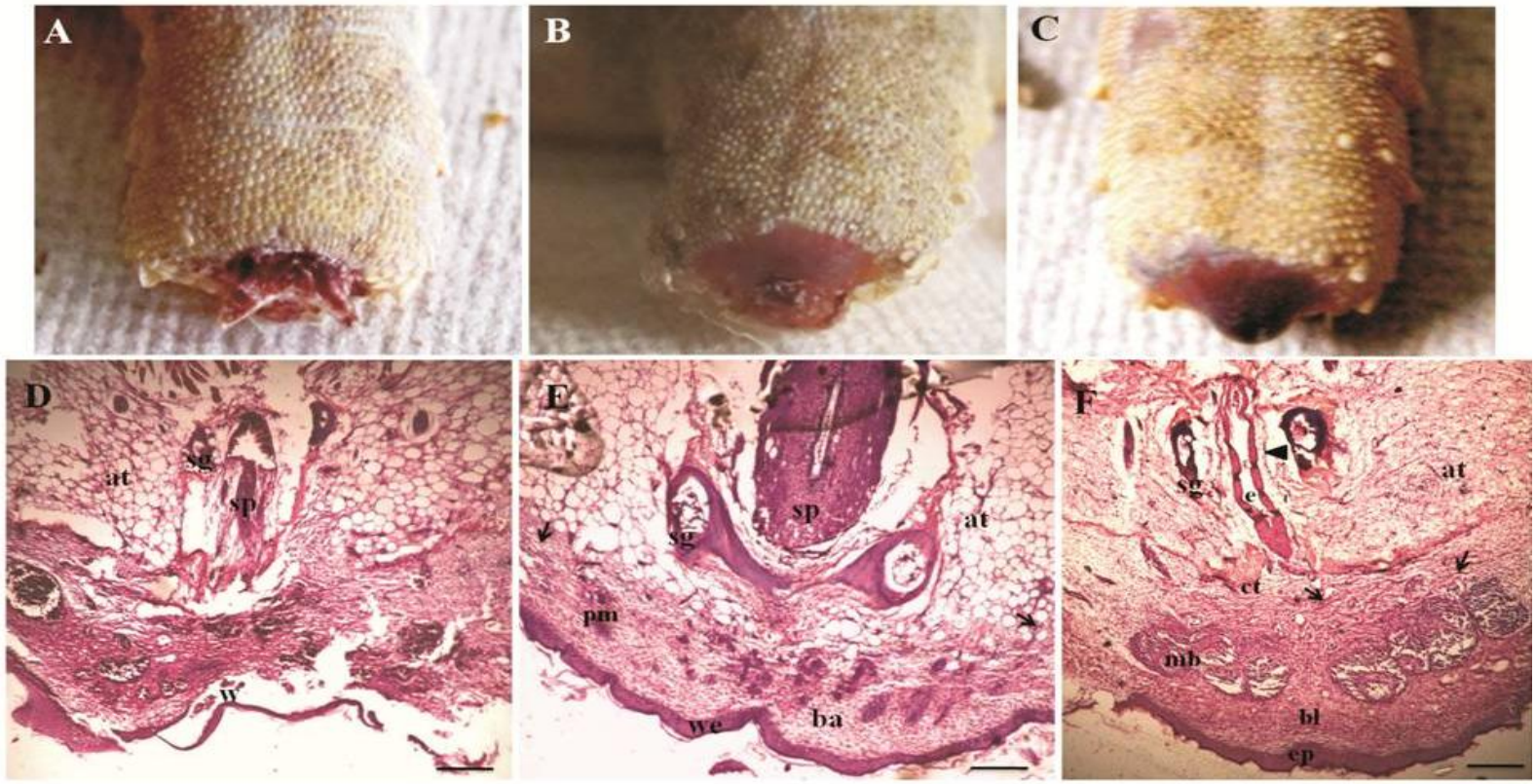
**Figure 1.6 Vehicle Control (DMSO treated) *H. flaviviridis*. Gross morphology of the regenerating tail in dorsal view (A-C) and histological details of the same (D-F)**.




**(A)** WE seen as a shiny smooth surface (5days post amputation (dpa)). **(B)** Blastemal cone 3-4mm in length (8dpa). **(C)** Differentiation stage with a 12-13mm long regenerate (13dpa). **(D)** A proper aec is prominent after wound healing. **(E)** Proliferating blastemal cells with regenerating muscles, ependyma and procartilage are evident during blastema. **(F)** Proper regenerating nervous, muscle and connective tissue along with a well formed epithelium are seen during differentiation.

aec- apical epithelial cap; at- adipose tissue; ba- cell accumulation prior to blastema formation; bl- blastema; c- cartilage; ct; connective tissue; e- ependyma; ep- epidermis; de- dermis; mb- muscle bundle; nt- neural tube; pc- procartilage; pm- promuscle; sg- spinal ganglion; rc- regenerating connective tissue; we- wound epithelium. Arrows indicate blood vessels. Scale bars: d-f = 1mm.

**Figure 1.7 SU5402 treated *H. flaviviridis*. Gross morphology of the regenerating tail in dorsal view (A-C) and histological details of the same (D-F).**




**(A)** Wound not healed in SU5402 treated animal (5days post amputation (dpa)). **(B)** A proper wound epithelium still not evident (9 dpa). **(C)** Blastema formation and proliferation is delayed in SU5402 treated animals with blastema evident only after 15dpa. **(D)** Wound not healed with traces of blood clot seen. **(E)** A thin WE seen along with accumulation of cells to form the blastema. **(F)** Improved stage of regeneration seen with an epithelium and regenerating ependyma, muscle bundles and traces of cartilage tissue (arrowhead).

at- adipose tissue; ba- cell accumulation prior to blastema formation; bl- blastema; ct; connective tissue; e- ependyma; ep- epidermis; mb- muscle bundle; pm- promuscle; sg- spinal ganglion; sp- spinal cord; w- wound yet to heal; we- wound epithelium. Arrows indicate blood vessels. Scale bars: d-f = 1mm.

# CHAPTER 2

## INVESTIGATING THE ROLE OF FGF2 SIGNALLING IN THE REGULATION OF TISSUE REMODELLING ACTIVITIES AND PROTEIN TURNOVER DURING EPIMORPHIC TAIL REGENERATION OF *HEMIDACTYLUS FLAVIVIRIDIS*

### INTRODUCTION

Epimorphic regeneration allows the functional regrowth of the lost appendage through strict growth controls and cell reprogramming occurring in adult tissues followed by sequential steps of cell differentiation and patterning leading to the faithful restoration of only the lost parts (Akimenko *et al*., 2003). During epimorphic regeneration, immediately after amputation, epidermal cells from the circumference of the lost part migrate to cover the exposed mesenchymal tissues (Bryant *et al*., 2002). This wound healing phase is achieved strictly through cell movement without cell division (Chalkley, 1954; Hay and Fischman, 1961). Subsequent blastema formation and repatterning cannot occur unless the amputation wound has healed, hence it is largely established that the wound-healing phase plays an important role in the initiation of the regenerative process (Campbell and Crews, 2008).

During lizard tail regeneration, following autotomy, keratinocytes proliferate from the living layers of the epidermis of proximally damaged scales, and migrate over the injured stump tissues, beneath the scab (Alibardi and Toni, 2005; Alibardi, 2010a). After injury, haemostasis serves as the initiating step and foundation for the healing process. Injured blood vessels vasodilate and the endothelium as well as nearby platelets activate the intrinsic part of the clotting cascade. Platelets release cytokines and growth factors that initiate the inflammatory response (Witte and Barbul, 1997). Immediately after the clot has formed, it attracts neutrophils and then monocytes, which are transformed into macrophages (Broughton *et al*., 2006). The macrophages are involved in phagocytosis of bacteria that may have been introduced into the wound during injury and also debris of damaged and injured cells that have undergone apoptosis. In addition, inflammatory mediators, released by macrophages and neutrophils, mainly growth factors are also necessary for the proliferation phase to occur. This involves initiation and propagation of granulation tissue formation, which is composed of fibroblasts and inflammatory cells, along with new capillaries embedded in a loose extracellular matrix of collagen, fibronectin, hyaluronic acid and glycosaminoglycans (Metcalfe and Ferguson, 2005). Fibronectin and fibrin create a provisional matrix that promotes migration of wound keratinocytes and formation of a soft mesenchyme over the tail stump cells, but once dermal/epidermal resurfacing is accomplished, its appearance is largely restricted to the basement membrane zone of the dermal-epidermal junction and of blood

vessels (Schultz and Wysocki, 2009). After migrating keratinocytes have covered the central part of the stump, the epidermis becomes stratified and thick, forming a wound epithelium. In this way macrophages mediate angiogenesis, synthesize MMPs to degrade the collagens of damaged tissues and, most importantly, stimulate re-epithelization of the wound, resulting in transition to the proliferative phase (Stocum, 1995; Broughton *et al*., 2006). Thus, extracellular matrix (ECM) is a dynamic environment and its macromolecules are critical for creating a conducive milieu for proper proliferation and migration of different cell types (Mathew *et al*., 2006). Recently, ECM has been recognized for its critical roles to maintain a special local environment for adult stem cells, stem cell niche, and ECM degradation involved in stem cell differentiation, activation, and/or release (William *et al*., 2007; Chen, 2010; Reilly and Engler, 2010).

It is known that early during regeneration, epidermis at the tip lacks a complete basement membrane (Korneluk *et al*., 1982; Young *et al*., 1985) and this condition is thought to be critical for early epithelial/mesenchymal signalling events involved in regeneration (Stocum, 1995). Also, relatively low and brief inflammatory response favours tissue regeneration (Tuchunduva *et al*., 2001; Wynn, 2008). In contrast, intense inflammatory reaction and the presence of a granulomatous reaction do not allow the establishment of a mesenchymal population, and the rapid formation of a continuous dense lamella in the basement membrane blocks any dermal-epidermal interactions to sustain the elongation of the lost part. These combined factors are characteristic of wound healing with scar formation (Ferguson and O'Kane, 2004; Alibardi, 2010).

All the above mentioned events of wound healing require the concerted action of various proteolytic enzymes. Indeed, tissue breakdown, cell movement, cell-cell and cell-matrix interactions and dedifferentiation can occur only if major alterations in the ECM take place (Tsonis *et al*., 1996). Timely degradation of ECM is an important feature of development, morphogenesis, tissue repair and remodelling which is precisely regulated under normal physiological conditions (Nagase *et al*., 2006). Various types of proteinases are implicated in ECM degradation, but the major enzymes considered to be primarily responsible for ECM turnover are the matrix metalloproteinases (MMPs), also called matrixins (Visse and Nagase, 2003).

MMPs were discovered as proteases capable of digesting collagen in the remodelling tissues of metamorphosing tadpoles (Gross and Lapiere, 1962; Fujimoto *et al*., 2006, 2007). They constitute a family of structurally related zinc-dependent proteolytic enzymes, currently with more than 20 members that are involved in degrading components of the ECM in the normal course of matrix turnover and renewal (Birkedal-Hansen *et al*., 1993). Members of the MMP family have been implicated in epithelial wound healing of the cornea, and the skin (Azar *et*

*al*., 1996; Fini *et al*., 1998; Ye and Azar, 1998), and in the regeneration of tissues including mouse muscle (Kherif *et al*., 1999), zebrafish heart and fin (Bai *et al*., 2005; Lien *et al*., 2006), and urodele appendages (Yang and Bryant, 1994; Miyazaki *et al*., 1996; Yang *et al*., 1999; Vinarsky *et al*., 2005).They are activated in regenerating limbs of newts and salamanders (Grillo *et al*., 1968; Yang and Bryant, 1994; Park and Kim, 1999) and also during inflammation phase of wound healing and function to clear inflammatory debris in mammals (Parks, 1999; Broughton *et al*., 2006). These proteolytic enzymes play a major role during development and morphogenesis. Improper regulation of these proteinases may result in pathologies such as arthritis, cancer, atherosclerosis, aneurysms, tissue ulcers, and fibrosis (Visse and Nagase, 2003).

MMPs are mainly produced by cells of mesenchymal and hematopoietic lineages (Birkedal-Hansen *et al*., 1993) and certain MMPs are expressed in specific embryonic and extra-embryonic tissues. Neutrophils (Schwartz *et al*., 1998) and macrophages infiltrating through vessel walls and vascular basement membrane are key producers of MMPs in wound healing (Yong *et al*., 1998). These cells have the potential to cleave and degrade type IV collagen in the basement membrane and collagens type I and III in the ECM. MMPs are not expressed, however, by stationary tissue fibroblasts (Hakkinen *et al*., 2000). It has been shown that MMPs play an important role in the breakdown of the provisional matrix formed after wounding by breaking down several components of the ECM, including type IV collagen, fibronectin, laminin, entactin and elastin, as well as clarifying the cellular and fibrillar debris that surround the wound bed (Baker and Leaper, 2000). This helps keratinocytes and fibroblasts to migrate through the basement membrane as well. The level of MMP-2 and MMP-9expression is up-regulated in the wound stroma due to migratory fibroblasts. MMP-2 and 9 are the only two proteases that degrade gelatin (denatured collagen), basal lamina collagens (Type IV, V, VII, X, XI), and the proteoglycan core protein. MMP-2 and 9, therefore, can breakdown the components of the basement membrane, including laminin, collagen type IV, and fibronectin (Yong *et al*., 1998). MMPs may also prevent basement membrane formation under wound epithelium, promoting epithelial/mesenchymal interactions (Yang *et al*., 1999), which are thought to be necessary to support blastemal growth (Tsonis, 1996). MMPs may release growth factors such as FGFs from the ECM to make them available to blastemal cells (Boilly *et al*., 1991; Hondermarck and Boilly, 1992). MMP activity may promote regeneration by preventing scar formation. This is supported by the observation that a scar-like epidermis is formed when newt limbs are treated with GM6001, an MMP inhibitor (Vinarsky *et al*., 2005).

Although MMPs are required for proper regeneration and may act to prevent a scar formation unregulated proteolytic activity may be detrimental to the regenerative process. The MMPs are specifically controlled at the transcriptional level, by activation of precursor zymogens,

cell-ECM interactions, and inhibition by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs) (Nagase and Woessner, 1999).

The inhibitory activity of TIMP-1 was discovered in the early 1970s in the form of a collagenase inhibitor in the media of cultured human skin fibroblasts (Bauer *et al*., 1975), human serum (Woolley *et al*., 1975), and in extracts of bovine cartilage and aorta (Kuettner *et al*., 1976). It was purified and found to be a 25-31kDa protein in 1979 (Vater *et al*., 1979; Welgus *et al*., 1979). To date, four members of the TIMP family have been identified, TIMP-1 to -4 (Matrisian, 1990; Nagase and Salvesen, 1993; Woessner and Nagase, 2000). TIMPs 1, 2, 3 are expressed by most cell types while TIMP-4 is found predominantly in brain tissue. Most TIMPs are secreted in to the intracellular space where they inhibit MMP activities. Each of the TIMPs can inhibit the activities of most of the MMPs (Woessner and Nagase, 2000), but affinities vary for different inhibitor-protease pairs (Brew and Nagase, 2010). Many investigations demonstrate that TIMPs can inhibit proliferation, invasion, and metastasis through inhibition of MMP activity (Sternlicht and Werb, 2001; Baker *et al*., 2002). In addition to their metalloproteinase inhibitory activity, TIMPs have various biological activities such as promoting cell proliferation, anti-angiogenic, pro-and anti-apoptotic and synaptic plasticity activities, many of which are independent of metalloproteinase inhibition (Brew and Nagase, 2010).

In many physiological processes, both MMPs and TIMP-1 are co-expressed, thereby producing a tightly regulated response required for optimal MMP activity (Gardner and Ghorpade, 2003). TIMP interactions form stoichiometric 1:1 complexes with the active form of MMPs (Woessner, 1991) and block MMP catalytic activity but have no effect against other types of proteinases (Gourevitch *et al*., 2003). Thus the balance between MMPs and TIMPs plays a key role in the regulation of the ECM degradative activities of the MMPs. Dysregulation of this interaction is a characteristic feature of pathological conditions which involve tissue destruction, e.g. tumour formation (Martorana *et al*., 1998), as well as matrix accumulation such as in diabetic nephropathy (McLennan *et al*., 2000).

FGF2 now certainly appears to influence wound healing and blastemal cell proliferation during *H. flaviviridis* tail regeneration since, its signalling inhibition delayed these processes and compromised with the quality of regeneration (Chapter 1). Hence, it is logical to presume that FGF2 might be involved in the initial cascades of events leading to wound healing and could be influencing the process of ECM turnover. In fact, FGF2 stimulates the expression of adhesion molecules on endothelial cells, thereby promoting the recruitment of inflammatory cells and potentiating the inflammatory process (Naik *et al*., 2003). Moreover, *in vitro* studies have shown that FGF2 is one of the important regulatory factors for ECM turnover via modulation of MMPs and TIMP secretion from subepithelial myofibroblasts

(SEMFs) (Yasui *et al*., 2004). Similarly, changes in MMP-2, 7 and TIMP-2 expressions are important processes of wound repair, which are closely related to the acceleration of wound healing by the application of FGF2 (Cheng *et al*., 2003).

Hence, in the current study, influence of FGF2 signalling on the matrix remodelling activities during reptilian epimorphosis was evaluated. This was achieved through analyzing activities of gelatinases (MMP-2 and MMP-9) by gelatin zymography and their immunohistochemical localization in the tail regenerates of control and SU5402 treated lizards. Further, by studying the levels of TIMPs, one can get an idea of the role of FGF2 in the interplay between MMPs and TIMPs and the process of matrix reorganization. TIMPs were assessed through reverse gelatin zymography.

Epimorphosis involves regulated orchestration of several physiological events, which inevitably requires large scale protein turnover. Protein metabolism gets extensively geared up in accordance with regressive (dedifferentiation) as much as with the progressive phases (blastema formation, differentiation and growth) of regeneration (Thornton and Bromley, 1973). This biosynthesis of proteins is one of the most important biochemical processes during regeneration and similarly down regulation of several proteins is also required. Several factors could be responsible for this and since, FGF2 undoubtedly influences epimorphosis, it is reasonable to deduce that it might influence this protein turnover also. Moreover, in an earlier work, *in loco* Anti-FGF2 treatment resulted in lower total protein levels in the regenerate of *H. flaviviridis* (Yadav, 2005). In the current study therefore, attempt was also made to understand the alteration if any, in the protein profile of the regenerate due to FGF2 signal inhibition during different stages of lizard tail regeneration.

## MATERIAL AND METHODS

### Animals

A total of eighty lizards of both sexes were selected for this study. The animals were acclimatized and maintained in the animal house as described elsewhere (Material and Methods). All experimental procedures were done under hypothermic anaesthesia. The animals were divided into two groups of thirty five animals each and were treated as described below. A separate group of 10 animals was used to collect normal intact tail samples and 24h post amputation samples for the zymography studies. At the end of the experiment, animals were rehabilitated for two months in standard housing conditions and released back to their natural habitat.

### Experimental Design and Drug Dosage

Group I: This group of animals served as control to the experimental groups and injected with vehicle (1%DMSO).

Group II: The animals were injected with SU5402 (0.7 mg/kg body weight).
Treatment started two days prior to amputation and was continued on every alternate day till the termination of the experiment. Control animals reaching the different stages of regeneration *viz*. wound epithelium (WE), blastema (BL) and differentiation (DF) were selected and the regenerates along with an intact tail segment were collected by inducing autotomy. The regenerates were then excised and processed further for evaluation of protein expression and for analysing activities of gelatinases as well as TIMPs. Regenerates of treated animals were collected on the day when the control animals reached the appropriate stages irrespective of whether treated animals attained the same stage or not. Samples of intact tail segment were also collected similarly.

**SDS-PAGE analysis of Proteins**

Briefly, protein content of tissue supernatants was determined using BCA assay kit (Genei Products, Merck, USA). Samples were prepared by boiling in sample loading buffer and equal amount of total protein was loaded and separated using 10% polyacrylamide gels. After electrophoresis, gels were stained with Coomassie Brilliant Blue-R250 stain and then destained (45:10:45 Methanol:Acetic acid:dd$H_2O$).

**Zymographic analysis of MMP-2 and MMP-9 activity**

In brief 7.5% SDS polyacrylamide gels with gelatin (5mg/ml) were prepared. Equal amount of protein samples were loaded using a non reducing loading buffer. After electrophoresis, gels were washed with 2.5% Triton X100 twice for 30 minutes each and rinsed in dd$H_2O$ followed by wash in incubation buffer (50mM Tris, 0.2M NaCl, 5mM $CaCl_2$, 0.02% $NaN_3$ and 0.02% Brij-35, pH 7.4-7.6) for 30 minutes and an18 hour incubation with the same at 37ºC. Gels were stained with Coomassie Brilliant Blue R-250 (Coomassie 0.5%w/v, Methanol 40%v/v, Acetic acid 10%v/v in dd$H_2O$) for 1-2 hours and then destained using acetic acid-methanol solution. Activity of MMPs could be observed as clear bands on a dark background.

**Immunohistochemical localization of MMP-2 and MMP-9**

Regenerates along with an intact tail segment were collected at different stages of regeneration. fresh frozen longitudinal cryostat sections (8-10µm) of these were taken on clean glass slides, fixed in cold acetone, air dried and rehydrated with PBST, then blocked using normal serum for 1-2 hours at RT. Sections were then incubated with appropriate primary antibody (1:300 dilution of Anti-MMP-2 or Anti-MMP-9) overnight inside a moist chamber at 4ºC. Next day, sections were washed with PBST and incubated with corresponding ALP-conjugated secondary antibodies (1:500dilution) for 2 hours at RT, washed and stained with substrate chromogen solution (BCIP/NBT) for 10-15 minutes. Sections were washed with PBS, mounted with a solution of PBS and glycerol (1:1) and

observed using a Leica DM2500 microscope. Negative control sections were incubated with PBS-BSA instead of the primary antibody (for details see Material and Methods). Mammalian antibodies against MMPs have been used considering their evolutionary conservation across vertebrate species (Yang *et al*., 1996).

**Reverse Zymographic analysis of TIMP**

In a few words, 15% SDS polyacrylamide gels with gelatin (2.5mg/ml) and crude MMP-2 (rhMMP-2, R&D Systems, USA) were prepared. Samples were loaded with a non reducing loading buffer and electrophoresed. Gels were then processed further in the same way as described for reverse zymography. After destaining, TIMP activities were detected as blue bands on a clear background. A regular SDS- PAGE gel for the samples was also run under identical conditions to discriminate between the TIMP and the protein bands, which will also get stained by the Coomassie blue.

**Determination of molecular weight and spot densitometry analysis**

Determination of Molecular weight of the zymographic and protein bands and spot densitometry analysis were done using AlphaEase FC software, stand alone for windows, version 4.0.034 (Alpha Innotech, San Leandro, CA). Molecular weight of the protein markers were manually added to the programme and the molecular weight of the query bands were determined based on the graph of the known marker bands. If a query band lay outside of the standard curve, its molecular weight was extrapolated in "Least Squares Fit" mode. The molecular weight data of the reference markers were stored in the software library for analysis of gels which were run without protein marker but with the similar electrophoresis condition. Similar areas were analyzed for the densitometry and quantitative comparison was made in all the bands of interest and the results were expressed in arbitrary units, which was calculated by integration of the intensity of each pixel over the spot area and normalized for the gel background.

## RESULTS

### MMP and TIMP activities during regeneration

An important observation made in the initial studies was that FGF2 is one of the key factors for a proper wound healing response as this process was undoubtedly subdued in the SU5402 treated lizards. The formation of the wound epithelium and the blastemal cone was delayed in these lizards as compared to control animals. Proper matrix remodelling through proteolytic activity is inevitable for successfully attaining these two hallmarks of regeneration. Since FGF2 was found to be crucial during this stage, its involvement in controlling the matrix reorganization was studied further by analyzing activities of gelatinases MMP-2 and 9 and their endogenous inhibitors- the TIMPs.

An obvious surge of gelatinase activity can be seen in response to amputation as could be observed in the zymography results for the intact tail sample and tail sample taken 24 hours post amputation (hpa). The activity of MMP-9 is especially seen to be high. High levels of both pro-enzyme and active forms were detected. Activity level of pro-enzyme MMP-2 was also high. Activity of these gelatinases was almost undetectable for the intact tail sample, indicating the role of these proteases immediately following tissue injury (Figure 2.1).

Results of gelatin zymography for control and SU5402 treated lizards during the different regenerative stages showed that FGF2 signalling inhibition definitely altered the gelatinase activity during the initial events of wound healing and blastemal formation. During wound healing stage, activity of both active and pro-enzyme forms of MMP-9 was prominent in the control lizards. High intensity band of pro-MMP2 was also evident at this stage (Figure 2.2). In the treatment group also, MMP-2 and MMP-9 activity areas could be observed, but MMP-2 activity was altered and a significantly lower activity of this gelatinase was detected. Gelatin degradation in the region of active MMP-9 was also affected significantly, although the region of its pro-enzyme form had comparable activity (Figure 2.3). However, the optical density results shown in Table 2.1 for MMP-9 accounted for its pro-enzyme form and these values for the treatment group were lower as compared to control values. Optical density values for MMP-2 were also significantly lower for treated animals compared to control during wound healing stage (Table 2.1). During blastemal proliferation, MMP-2 was the gelatinase found to be significantly active in the control animals although slight MMP-9 activity was also detected at a higher protein concentration (Figure 2.4). Treatment group recorded a lighter band of MMP-2 gelatinase during this stage and almost negligible MMP9 activity (Figure 2.5). The optical density values also reflected the same results as above (Table 2.2). Activity of gelatinases in control group during late proliferation was found to be low. Nevertheless, MMP-2 was the evident gelatinase active at this stage as well. Slight gelatin degradation was also observed in the region of MMP-9 activity (Figure 2.6). The treatment group recorded a comparable MMP-2 activity at this stage as the control group. Like the control group, MMP-9 gelatin degradation was not prominent (Figure 2.7). The optical density results for this stage showed comparable values for both the groups (Table 2.3).

Thus, higher activity of these gelatin proteases was evidently required during initial regenerative stages however with the progress of regeneration their activities were found diminishing. Of the two proteases, MMP-9 activity was required at a greater extent during initial wound healing, whereas the role was taken over by MMP-2 following wound healing during blastema formation, although appreciable activity of MMP-2 pro-enzyme form was detected during wound healing. An obvious influence of FGF2 in regulating the MMP-2 activity during reptilian epimorphosis could be deduced since MMP-2 activity was

significantly reduced in the FGF2 signal inhibited animals during both these initial regenerative stages. Although, from the zymograms, it can be observed that FGF2 inhibition did not significantly alter pro-MMP-9 activity, optical density of the activity bands definitely indicated towards a better MMP-9 activity in the control animals. Nevertheless active form of this proteinase was definitely affected by FGF2 inhibition. Treatment group recorded a comparable activity of gelatinases as the control group after a particular wound healing response was achieved. Possibly matrix degradation activities were delayed due to impaired gelatinase activity in the SU5402 treated lizards and this was reflected in the time required to complete wound healing and blastemal proliferation and hence, the observed delay in regeneration for the treatment group (chapter 1).

To supplement the above study and to further understand the tissue distribution as well as activity levels MMPs during different stages, immunohistochemical localization of gelatinases was also done. Results obtained are in accordance with the above study. Significant activity of both gelatinases could be observed in the control animals during wound healing. MMP-2 localization was prominent during blastemal and differentiation stages as in accordance with the zymogram results. Main sites of localization were the wound epithelium, especially the dermis and blastemal mesenchyme during wound healing. In addition to these, the developing ependyma and supporting cartilage tube were positively labelled during later stages (Figure 2.12). However, a slight dispersed localization was seen in the entire regenerate as well. Comparatively a lighter signal of MMP-2 activity was observed for the SU5402 treated lizards during wound healing and blastemal stages. Better labelling of MMP-9 was observed at 6 days post amputation for this group (Figure 2.13).

Influence of FGF2 on the activity of endogenous MMP inhibitors, TIMPs was also studied through reverse zymography. Apart from inhibiting MMPs, they also affect cell proliferation and cell survival independently of the mechanism of MMP inhibition, indicating their importance for a process like regeneration. So, it would be of significance to know whether their activity during reptilian regeneration is dependent on FGF2 signalling. Reverse zymograms did show the presence of bands in the region of 20-30 kDa range. However, since clear demarcation of different TIMPs could not be observed, conclusive remarks about specific TIMP activity cannot be made. Nevertheless, proteolytic degradation was definitely accompanied by a concomitant activity of TIMPs, immediately following amputation. But there were no significant qualitative differences in TIMP activity in intact tail and in tail sample 24hpa, although the 24hpa lane showed better activity (Figure 2.8). It is possible that under normal conditions, MMP activity is kept in check by these TIMPs and their level initially rises to regulate the intense degradative activities of MMPs following amputation. Among the regenerative stages of control animals, TIMP activity was lowest at wound epithelium stage and its levels became comparable to that of 24hpa at the differentiation stage

(Figure 2.9). This can be correlated with the gelatinase activity observed earlier. TIMP activity for the treatment group was definitely less intense as compared to the control group, with comparatively better bands observed only during blastema stage (Figure 2.10). The bands detected in the reverse zymograms were conclusively TIMP bands as a normal PAGE run under identical conditions did not show the presence of any bands in the molecular weight range of 20-30kDa (Figure 2.11).

Clearly, combined results of the above studies indicate towards the role of FGF2 in maintaining the optimal balance between MMP and TIMP activities required for the formation of a proper regenerate. Altered MMP and TIMP levels in SU5402 treated lizards may lead to improper ECM remodelling activities and subsequently poor quality of regenerate. Evidently the event of matrix reorganization during reptilian epimorphosis definitely requires proper FGF2 signalling.

**SDS-PAGE Protein profile of tail regenerates**

Since the results obtained so far pointed towards the influence of FGF2 on the initiation and progression of tail regeneration in *H. flaviviridis*, it was thought worth to find the protein alteration occurring during regeneration when FGF2 signalling is blocked. The results obtained were for one-dimensional gel electrophoresis and the PAGE profile during wound healing and blastema stages of the tail point towards a possible involvement of FGF2 in regulating protein turnover. During these stages, several polypeptides of identical mobility were found in control and SU5402 treated tail samples, although absence of some bands was observed for the treated group. Even, the polypeptide bands which were common for both the groups also varied in their intensity with the control groups showing abundance of most of the proteins (Figure 2.14-2.16).

At the WE stage, prominent polypeptide bands with molecular masses of 159.81, 92.18, 81.63, 59.67 and 36.03 kDa were observed in the control group. However, the intensity of these bands was significantly less for the treatment group with an exception for the 81.63 kDa band, which showed comparable intensity. Similarly, polypeptides with molecular masses of 189.51, 117.22, 102.99, 53.83 and 12.98 were observed in control groups but these were comparatively less prominent, nevertheless, intensity of these bands in the treatment group was again low as compared to control group. Absence of certain bands with molecular masses of 141.01, 41.02, 31.64, 26.05 kDa in the treatment group was also observed (Figure 2.14, Table 2.4).

During blastema stage, polypeptides with similar molecular masses as the WE stage were observed. Bands of molecular masses 159.83, 81.61, 59.88, 30.3 kDa were prominent in the control group. In the treatment group, intensity of these bands was greatly affected, with an

exception of the 81.61 kDa band. Also absence of bands with molecular mass 127 and 19.65 kDa was evident in the treatment group. Other lesser prominent bands were also seen in the range of 30-160 kDa, intensity of which for the treatment group was evidently variable from that seen in the control group (Figure 2.15, Table 2.5). Polypeptides in the molecular weight range of 13-160 kDa were observed at the differentiation stage, however, the intensity and presence of bands observed was comparable for both the experimental groups at this stage (Figure 2.16, Table 2.6). Evidently, FGF2 is not only involved in regulating proteolytic activity, but also the expression of several proteins during *H. flaviviridis* tail regeneration.

**DISCUSSION**

FGF2 significantly governs the wound healing and blastemal accumulation processes during *H. flaviviridis* tail regeneration. Both of these events depend upon a regulated degradation of the ECM. This ECM modification in turn promotes cell survival, proliferation, differentiation, and patterning. Indeed the ECM is remodelled extensively during limb regeneration (as are the muscles, blood vessels, nerves and bones at the amputation site) (Madlener, 1998). Proteolytic degradation of ECM is the key to inflammatory response, cell-cell and cell-matrix interactions, and cell migration leading to the formation of the WE, the structure that provides the signals required for subsequent proliferation (Campbell and Crews, 2008). Degradation of ECM also plays an important role in cell migration towards the underlying mesenchymal layer to form blastema. The major group of proteolytic enzymes responsible for ECM degradation during processes that require ECM turnover, including regeneration, are the matrix metalloproteinases (MMPs). MMPs participate in regenerative response across a wide range of animals including hydra foot and head regeneration (Leontovich *et al*., 2000; Shimizu *et al*., 2002), zebrafish fin regeneration (Bai *et al*., 2005), amphibian limb regeneration (Vinarsky *et al*., 2005), and mouse liver regeneration (Alwayn *et al*., 2008).

MMP-1, 2, 3, and 9 have all been implicated in corneal wound healing and ulceration (Matsubara *et al*., 1991; Fini *et al*., 1998; Ye and Azar, 1998). MMP-9 in particular is upregulated very early in the wound healing phase during amphibian regeneration and could be an important factor produced by the WE that initiates the dedifferentiation of the mesenchymal tissues (Yang and Bryant, 1994). Similarly Miyazaki *et al*. (1996) found that NtMMP-9 expression is up-regulated during newt limb regeneration. MMP-9 is also involved in mouse muscle regeneration by activating satellite cells (Kherif *et al*., 1999). It has been reported that MMP-9 expression in the epidermis is associated with keratinocyte migration and is not needed for downstream WE function (Yang *et al*., 1999; Satoh *et al*., 2008)

Thus MMP activity forms the basis for ECM turnover and subsequent regeneration. Since, FGF2 was found to influence the formation of the wound epithelium in the studied animal

model it was worth knowing its role in regulating these proteolytic enzymes. Hence, activity levels of the gelatinases MMP-2 and 9 were evaluated in control and FGF2 signal inhibited lizards. Results of zymography revealed that FGF2 signalling is required for proper proteolytic remodelling of the ECM, as FGF2 inhibition significantly reduced gelatinase activity, particularly MMP-2 and active MMP-9 during wound healing and blastema stages, during which gelatinase activity is required the most. Impaired gelatinase activity may be the reason for delayed cell migration, reepithelialization and WE formation observed for the inhibitor (SU5402) treated lizards during morphometric studies (chapter 1). Delayed wound healing must also have retarded transition to the proliferative phase and hence, the observed delay in attaining the blastema stage can be correlated as well.

There are several reports suggesting the positive influence of FGF2 on regulating MMP activity. FGF2 is known to stimulate secretion of matrix degrading proteins in both endothelial and smooth muscle cells (Tsuboi *et al*., 1990; Kenagy *et al*., 1997). FGF2 also stimulates endothelial cell migration, pericyte attraction and matrix deposition by an increase in production of MMPs and VEGF (Presta *et al*., 2005). According to a study, the adenoviral oncogene E1A regulates FGF2 production and determines acquisition of a proangiogenic phenotype in bovine aortic endothelial cells (BAEC). BAEC transfected with wild type E1A showed an increase in MMP-9 expression and anti-FGF2 neutralizing antibodies reduced the amount of MMP-9 released by the transfected BAEC (Giampietri *et al*., 2000). Stimulation of bovine nucleus pulposus cells cultured in monolayer with FGF2 augments the production of MMP-13 (potent matrix degrading enzyme) at the transcriptional and translational level in a dose-dependent manner (Li *et al*., 2008). FGF2 is also known to increase MMP-9 mRNA levels in mouse and rabbit osteoclasts. This may be important for the migration of osteoclasts through the unmineralized osteoid to reach the mineralized bone surface (Chikazu *et al*., 2000). FGF2 mediates epithelial-mesenchymal interactions of peritubular and Sertoli cells in rat testis and this is known to involve a strong induction of MMP-9 and a weak induction of MMP2 in a coculture system (Ramy *et al*., 2005). It has been reported that curcuminoids inhibit the angiogenic response stimulated by FGF2, including expression of MMP-9 in the process (Mohan *et al*., 2000). Jenniskens *et al*. (2006) studied the effects of FGF2 on biochemical and functional modulation of newly formed cartilage collagen network *in vitro* and reported that FGF2 mainly decreased collagen deposition and this was accompanied by a significant increase in the level of MMPs. Further, FGF2 is known to affect migration of a variety of cell types (Ornitz and Itoh, 2001), and this may be indirectly due to its influence on MMP activity.

MMPs also influence angiogenesis (Heissig *et al*., 2003) and play an essential role in liberating growth factors and cleaving ECM proteins to reveal regions that can activate growth factor receptors (Mott and Werb, 2004). Direct physicochemical interactions with the

ECM enhance or inhibit the activity of many growth factors and are required for the activity of others, including FGF2. Indirect interactions between the ECM and growth factors occur via integrins-receptors responsible for cellular adherence which is a requisite for cellular responses to growth factors. Similar interactions are suggested from a study which reported that FGF2 release from the lens capsule by MMP-2 maintains lens epithelial cell viability (Tholozan *et al*., 2007). This directs towards a positive feedback loop between growth factors and MMPs and suggests that ECM-growth factor interactions are bidirectional and interdependent (Schultz and Wysocki, 2009).

Low MMP expression in muscle cells underlying histolysis versus strong staining in cartilage, bone and epidermis have been observed during limb regeneration of Mexican axolotl (Monaghan, 2009) Similar observations have been made during limb regeneration for MMP3/10b and MMP9 in the Japanese newt (Kato *et al*., 2003) and MMP9 in larval axolotls (Yang *et al*., 1999), but strong expression was observed in blastema cells for MMP9 and collagenase in the American newt (Vinarsky *et al*., 2005). Possibly the WE, bone, and inflammatory cells secrete the necessary MMPs to promote muscle histolysis and blastema formation (Monaghan, 2009). In the current study also a similar localization pattern of gelatinases has been observed with main sites of activity being the wound epithelium, specifically the dermal region, blastemal mesenchyme and cartilage during later stages of regeneration.

Further, MMP proteolytic activities need to be regulated for controlled ECM degradation, since unregulated MMP activity can be unfavourable for regeneration. Mechanisms for maintaining proper MMP activity during regeneration include the well established regulation of mRNA levels (Yang and Bryant, 1994; Vinarsky *et al*., 2005) as well as the post-translational modification, secretion, activation, pericellular location, and catabolism and clearance of MMP proteins (Sternlicht and Werb, 2001). Another way of inhibiting their activity is by way of endogenous TIMPs. These inhibitors influence regeneration not only by inhibiting MMP activity but also influence cell survival and proliferation. TIMP-1and 2 have been shown to act as mitogens on a variety of human, bovine, and mouse cell types, including erythroid precursor cells, keratinocytes, fibroblasts, smooth muscle cells, endothelial cells and chondrocytes (Gasson *et al*., 1985; Bertaux *et al*., 1991; Nemeth and Goolsby, 1993; Hayakawa *et al*., 1994; Yamashita *et al*., 1996; Wang *et al*., 2002). These dual functions of TIMP are independent of each other, given that point mutations that abolish the proteolytic inhibitory function of human TIMP-1 do not ablate its mitogenic activity (Chesler *et al*., 1995). Human TIMP-1 has also been shown to be a cell survival factor for several cell types (Murphy *et al*., 2002; Yoshiji *et al*., 2002), as well as an enhancer or inhibitor of bone resorption depending on whether TIMP-1 concentrations are low or high, respectively (Sobue

*et al*., 2001). These reports indicate towards the requirement of TIMPs to be as important as MMP activities.

Since, FGF2 influences gelatinase activity during *H. flaviviridis* tail regeneration, regulation of its inhibitor activity must also be partly under FGF2 control. Indeed, results of reverse zymography showed that the TIMP levels observed for control lizards differ from those of SU5402 treated ones. Altered TIMP activity may also be one of the factors responsible for improper regenerative events observed in FGF2 signal inhibited animals. In addition to affecting MMP regulation, the potential of TIMPs to influence cell proliferation may also be impaired by SU5402. This indicates that a proper FGF2 signal is absolutely essential for optimal MMP-TIMP balance characteristic of appropriate regeneration. TIMP regulation by FGF2 is known in other systems as well. Enhanced TIMP-1 expression by FGF2 has been observed in several cell types (Overall, 1994). Similarly, FGF2 also increases TIMP-1 expression, represses TIMP-2, but does not influence TIMP-3 expression in vascular smooth muscle cells during collagen fibre modelling following vascular injury indicating towards differential regulation of TIMPs by FGF2 (Pickering *et al*., 1997). FGF2 may also be one of important regulatory factors for ECM turnover via modulation of MMP and TIMP secretion from subepithelial myofibroblasts, whereby it stimulates MMP1, MMP3 and TIMP1 secretion, but does not affect MMP2 or TIMP2 secretion (Yasui *et al*., 2004). Altogether, results of the current study and supporting reports show that matrix reorganization event during *H. flaviviridis* tail regeneration was evidently under the influence of FGF2 which is required for optimal proteolytic as well as TIMP activity and altered levels of both in SU5402 treated lizards provide the required evidence supporting this notion.

Further, a process like regeneration requires concerted action of several gene families such as Wnt, shh, FGF, BMP, Notch, etc. (for review see Stoick-Cooper *et al*., 2007). Regulation of differential expression of these genes as well as their gene products, which are proteins, forms the basis for the several events of regeneration. The protein expression pattern was found to alter during different stages of regeneration in *H. flaviviridis* (Sharma *et al*., 2011). FGF2 being known as a major factor controlling epimorphosis at several stages, inhibition of this signalling pathway may lead to a variation in the expression of proteins. Hence, an SDS-PAGE analysis of proteins of the tail regenerates was done in control and SU5402 treated lizards. Proteins of identical mobility were found in the experimental groups during all stages. Results however, clearly showed that FGF2 signalling inhibition led to downregulation of several proteins in the treated animals as compared to those in the control lizards at the wound epithelium and blastema stages. Absence of certain bands in the FGFR1 inhibited lizards during these stages was also noticeable. However, during the differentiation stage, no significant differences in the relative intensity of the polypeptides between the two groups were evident.

One of the protein bands had a similar molecular weight as that of MMP-2 (66kDa) which was observed in abundance in the control animals during initial stages but found downregulated (in terms of optical intensity) in the treated lizards. This can be correlated to the results obtained earlier in the zymography study. Besides, prominent differences in the intensity of polypeptide bands with molecular masses around 159, 102, 59 and 36 kDa during wound epithelium and blastema stages between control and treatment groups were also observed. Downregulation of several other proteins in the treatment group during initial stages was also evident from the results of optical density analysis. Variations in the abundance of the polypeptides as well as absence of certain bands altogether could be attributed to the absence of proper FGF2 signalling in the SU5402 treated lizards. Impaired regeneration observed in this group could be due to the down regulation of several proteins being controlled by FGF2, essentially pointing towards its requirement for a proper regenerative response. Nevertheless, a proper conclusion about the extent of influence of FGF signalling on protein turnover could be drawn only through a detailed peptide analysis, identifying the proteins involved by incorporating 2D gel electrophoresis followed by MALDI-TOF MS/MS. This was beyond the scope of the present study and will be performed in one of the upcoming projects. The current study of SDS-PAGE however, will act as a formidable baseline data for the said future course of work.

The results from the above study consolidate the fact that FGF2 signalling is inevitable for the proper conduct of the early events of tail regeneration in lizards. However, its role in regulating several cellular events also needs to be elucidated for the proper consolidation of the present notion. Hence, in the following chapter influence of FGF2 signalling on some of the important cellular events during epimorphosis *viz*., apoptosis, blastemal cell proliferation and angiogenesis were explored. The study was also extended to understand the cross-talk, if any, between FGF2 and COX-2, the inflammatory mediator, which is identified as another important factor that regulate epimorphosis in *H. flaviviridis* (Sharma and Suresh 2008). Such a study would of help in gaining a broader, if not comprehensive, insight into the closely coordinated regulation of reptilian regeneration.

**Table 2.1: Densitometric analysis of gelatinolytic activity in control and SU5402 treated animals at wound epithelium (WE) stage.**

| GELATINASE | CONTROL | | | SU5402 TREATED | | |
|---|---|---|---|---|---|---|
| | Lane 1 | Lane 2 | Lane 3 | Lane 1 | Lane 2 | Lane 3 |
| **MMP-2** | 22374[@] | 29726 | 14667 | 3070 | 2964 | 5027 |
| **MMP-9** | 22365 | 15294 | 18388 | 13627 | 11695 | 11051 |

**Table 2.2: Densitometric analysis of gelatinolytic activity in control and SU5402 treated animals at blastema (BL) stage.**

| GELATINASE | CONTROL | | SU5402 TREATED | |
|---|---|---|---|---|
| | Lane 1 | Lane 2 | Lane 1 | Lane 2 |
| **MMP-2** | 23530[@] | 17126 | 9379 | 7016 |
| **MMP-9** | 7775 | 9904 | 3279 | 4870 |

**Table 2.3: Densitometric analysis of gelatinolytic activity in control and SU5402 treated animals at Differentiation (DF) stage.**

| GELATINASE | CONTROL | | SU5402 TREATED | |
|---|---|---|---|---|
| | Lane 1 | Lane 2 | Lane 1 | Lane 2 |
| **MMP-2** | 11914[@] | 12120 | 11456 | 9158 |
| **MMP-9** | 3419 | 2654 | 4166 | 4363 |

@Densitometry values for zone of clearance are expressed in arbitrary units calculated on the basis of area enclosed by the region in pixels together with band intensity. Lanes represent samples with different protein concentrations. In order to minimize background error a total of 5 randomly selected background areas were normalized.

**Table 2.4. The protein profile in the tail of control and SU5402 treated animals at wound epithelium stage.**

| MOLECULAR WEIGHT | LANE 1 (Optical Density expressed in arbitrary units) | LANE 2 (Optical Density expressed in arbitrary units) |
|---|---|---|
| **189.51 kDa** | 49962 | 41289 |
| **159.81 kDa** | 74648 | 44298 |
| **141.01 kDa** | 66438 | - |
| **117.22 kDa** | 65059 | 47891 |
| **102.99 kDa** | 74001 | 42831 |
| **92.18 kDa** | 71598 | 46027 |
| **81.63 kDa** | 80080 | 69953 |
| **59.67 kDa** | 66411 | 59996 |
| **53.83 kDa** | 70871 | 55410 |
| **41.02 kDa** | 62954 | - |
| **36.03 kDa** | 76440 | 53408 |
| **31.64 kDa** | 61388 | - |
| **26.05 kDa** | 55983 | - |
| **12.98 kDa** | 44210 | 36387 |

The entire protein profile subjected to densitometric analysis is expressed in arbitrary units. LANE-1 corresponds to control; LANE-2 corresponds to animals treated with SU5402. The blank cells indicate absence of specific protein bands.

**Table 2.5. The protein profile in the tail of control and SU5402 treated animals at blastema stage.**

| MOLECULAR WEIGHT | LANE 1 (Optical Density expressed in arbitrary units) | LANE 2 (Optical Density expressed in arbitrary units) |
|---|---|---|
| **159.83 kDa** | 53359 | 34412 |
| **141.22 kDa** | 37220 | 33659 |
| **127 kDa** | 54183 | - |
| **115.22 kDa** | 40975 | 37781 |
| **101.80 kDa** | 61241 | 44086 |
| **92.37 kDa** | 67101 | 51340 |
| **81.61 kDa** | 91719 | 78707 |
| **73.39 kDa** | 62215 | 58482 |
| **59.88 kDa** | 65059 | 62054 |
| **53.85 kDa** | 59594 | 58687 |
| **48.86 kDa** | 55116 | 53445 |
| **37.8 kDa** | 45369 | 43694 |
| **30.3 kDa** | 50202 | 48119 |
| **19.65 kDa** | 41117 | - |

**Table 2.6. The protein profile in the tail of control and SU5402 treated animals at differentiation stage.**

| MOLECULAR WEIGHT | LANE 1 (Optical Density expressed in arbitrary units) | LANE 2 (Optical Density expressed in arbitrary units) |
|---|---|---|
| **158.65 kDa** | 11073 | 20608 |
| **114.63 kDa** | 46988 | 37474 |
| **97.40 kDa** | 46809 | 37537 |
| **81.53 kDa** | 182374 | 89694 |
| **68.43 kDa** | 61052 | 57369 |
| **59.75 kDa** | 85721 | 56558 |
| **49.85 kDa** | 57445 | 51533 |
| **36.32 kDa** | 39342 | 24066 |
| **27.93 kDa** | 57227 | 52101 |
| **24.17 kDa** | 30714 | 33812 |
| **13.18 kDa** | - | 17218 |

The entire protein profile subjected to densitometric analysis is expressed in arbitrary units. LANE-1 corresponds to control; LANE-2 corresponds to animals treated with SU5402. The blank cells indicate absence of specific protein bands.

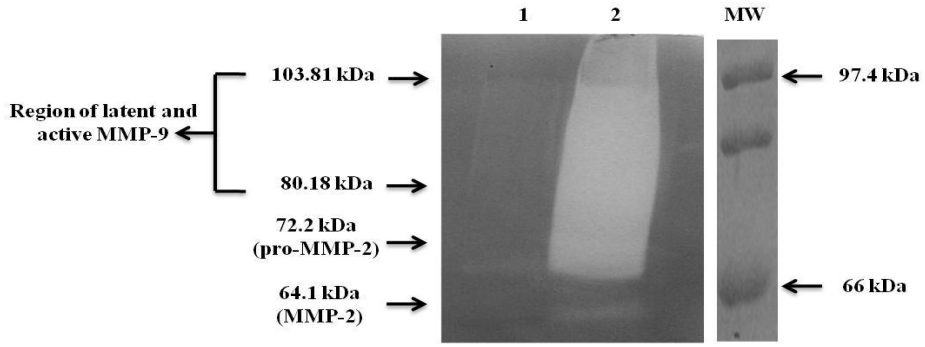



**Figure 2.1 Gelatinase activity in normal intact tail sample (Lane 1) and 24h post amputation sample (Lane 2). MW corresponds to molecular weight marker.**

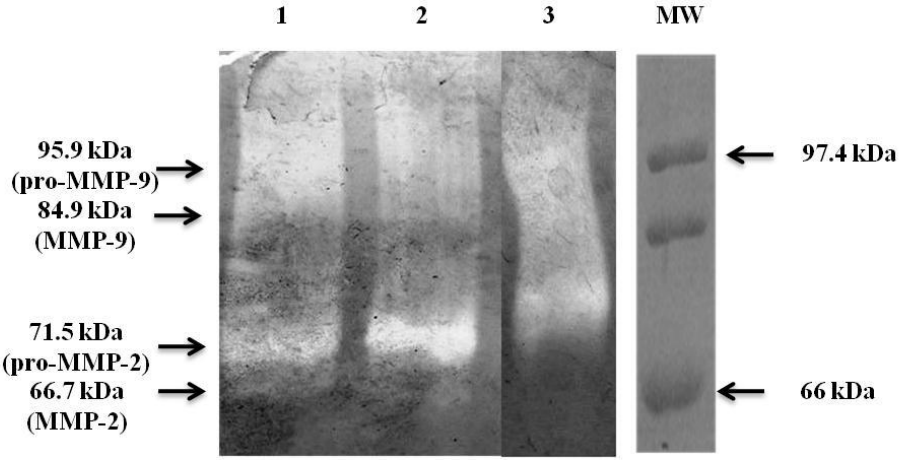



**Figure 2.2. Gelatinolytic activity of MMP-2 and MMP-9 in control animals at wound epithelium (WE) stage. Lanes 1-3 correspond to samples with different protein concentrations. Lane MW corresponds to molecular weight marker.**

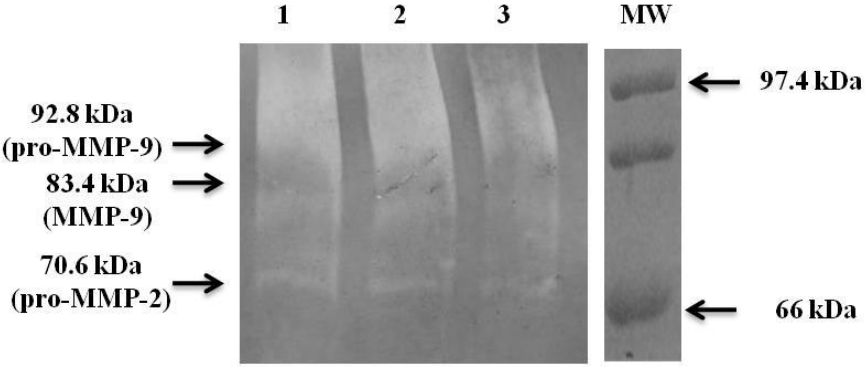



**Figure 2.3. Gelatinolytic activity of MMP-2 and MMP-9 in SU5402 treated animals at 6 dpa (days post amputation). Lanes 1-3 correspond to samples with different protein concentrations. Lane MW corresponds to molecular weight marker.**

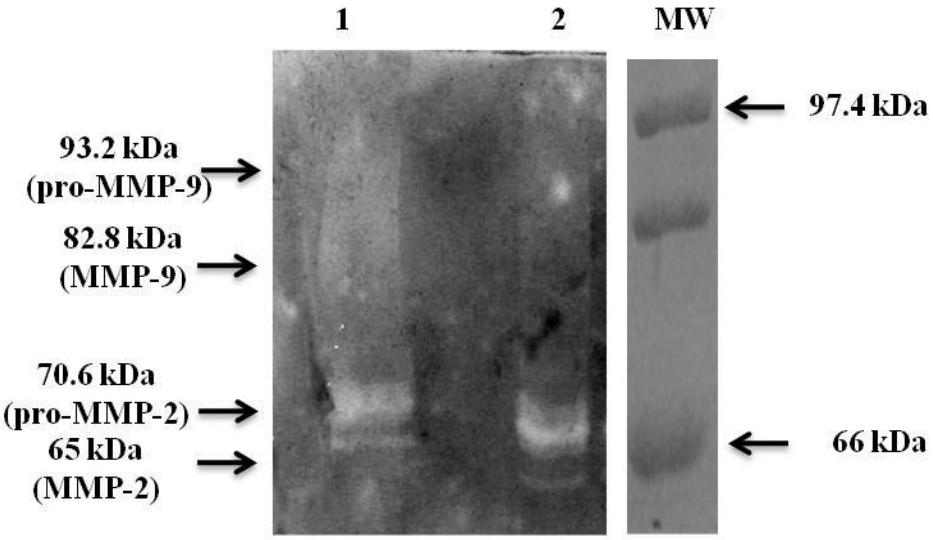



**Figure 2.4. Gelatinolytic activity of MMP-2 and MMP-9 in control animals at blastema (BL) stage. Lanes 1, 2 correspond to samples with different protein concentrations. Lane MW corresponds to molecular weight marker.**

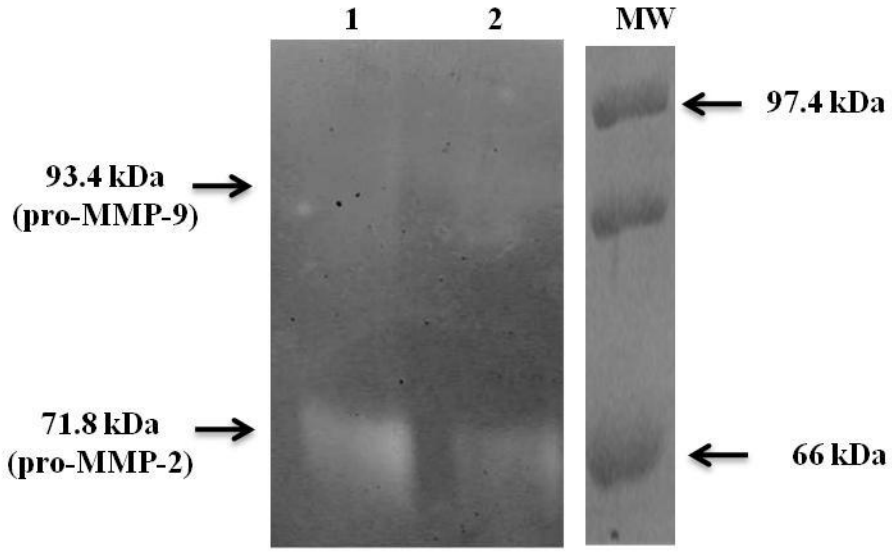



**Figure 2.5. Gelatinolytic activity of MMP-2 and MMP-9 in SU5402 treated animals at 9 dpa. Lanes 1, 2 correspond to samples with different protein concentrations. Lane MW corresponds to molecular weight marker.**

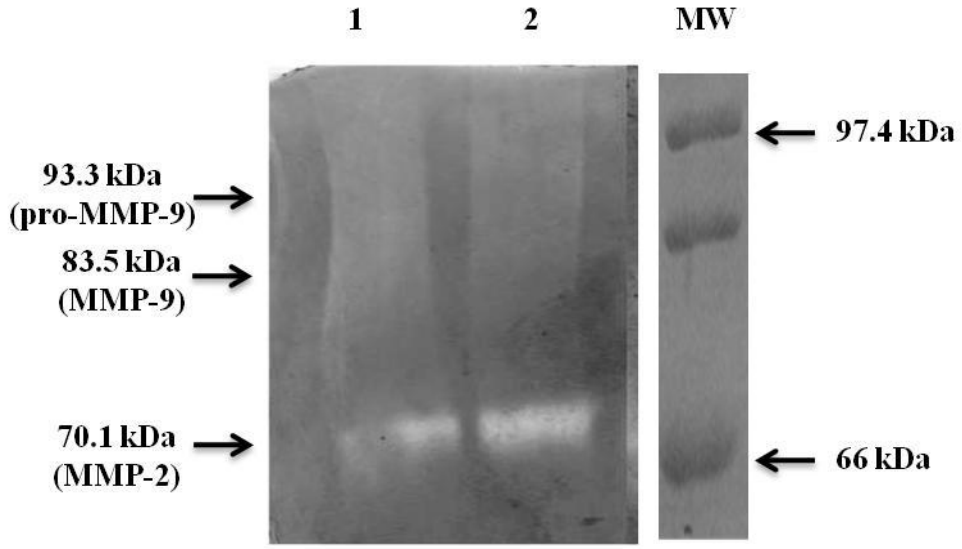



**Figure 2.6. Gelatinolytic activity of MMP-2 and MMP-9 in control animals at differentiation (DF) stage. Lanes 1, 2 correspond to samples with different protein concentrations. Lane MW corresponds to molecular weight marker.**

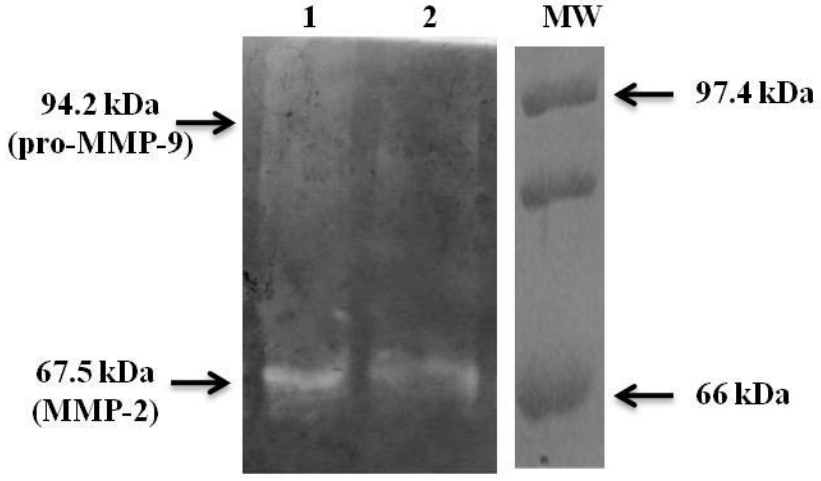



**Figure 2.7. Gelatinolytic activity of MMP-2 and MMP-9 in SU5402 treated animals at 13 dpa. Lanes 1, 2 correspond to samples with different protein concentrations. Lane MW corresponds to molecular weight marker.**

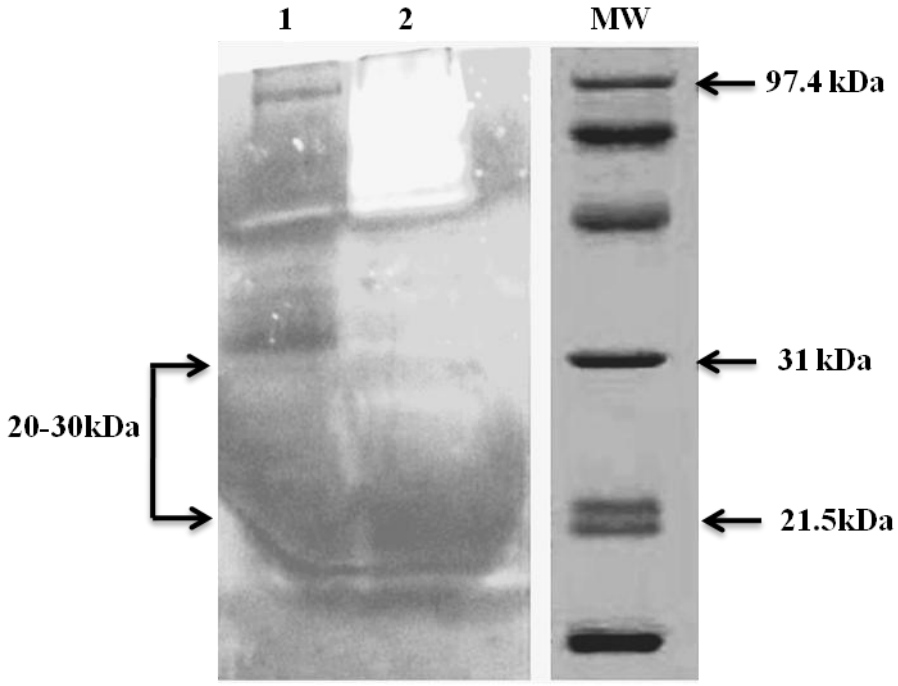



**Figure 2.8. Reverse zymogram showing TIMP activity (between 20-30kDa) in intact tail (Lane 1), and 24h post amputation (Lane 2) samples. MW corresponds to molecular weight marker.**

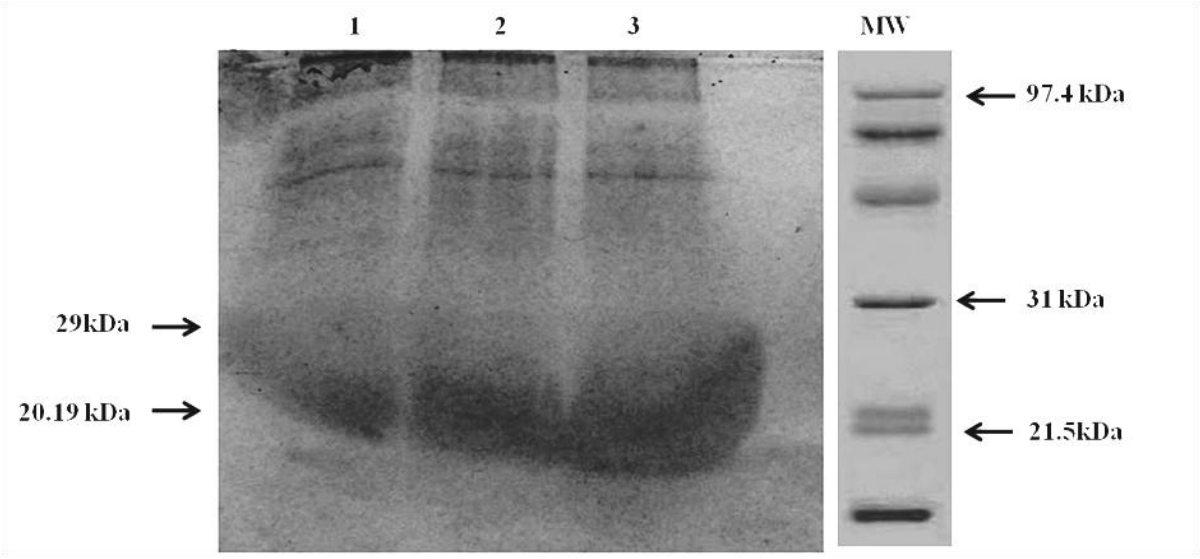



**Figure 2.9. Reverse zymogram showing TIMP activity (between 20-30kDa) in control animals at wound epithelium (Lane 1), blastema (Lane 2), and differentiation (Lane 3) stages. MW corresponds to molecular weight marker.**

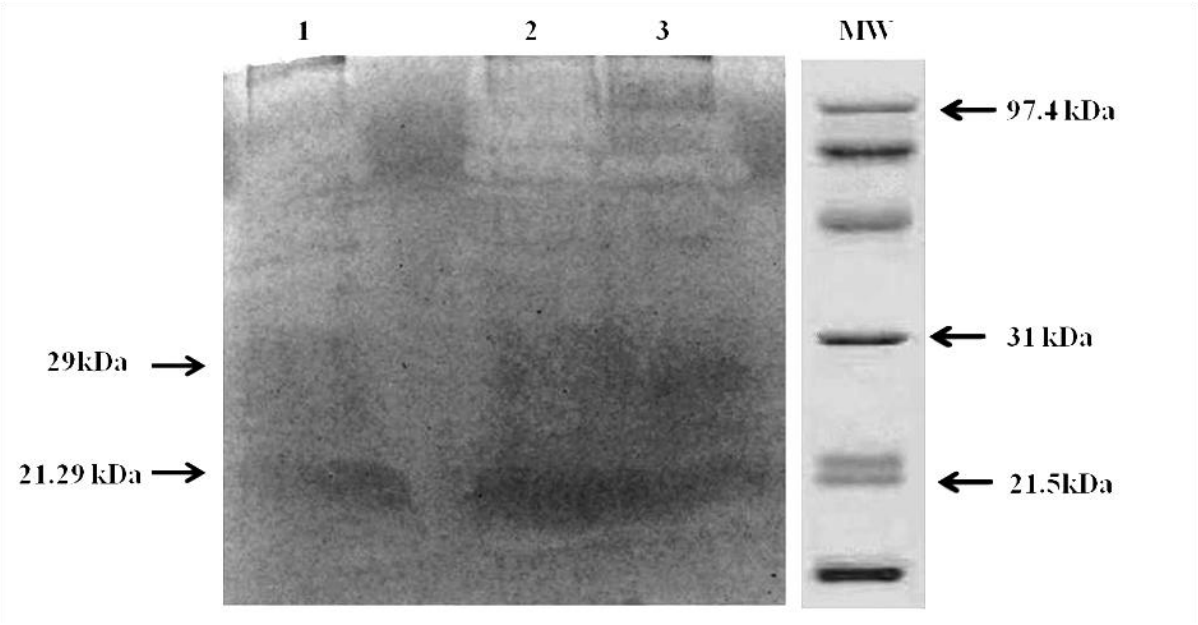



**Figure 2.10. Reverse zymogram showing TIMP activity (between 20-30kDa) in SU5402 treated animals at 6 dpa (Lane 1), 9 dpa (Lane 2), and 13 dpa(Lane 3) stages. MW corresponds to molecular weight marker.**

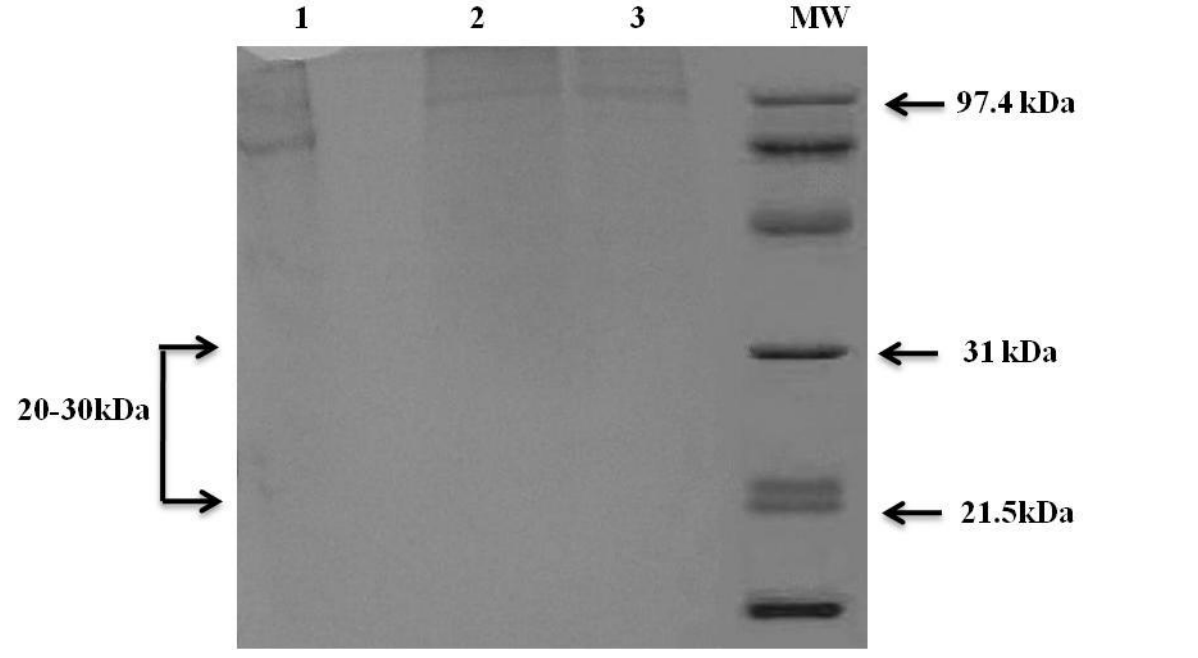
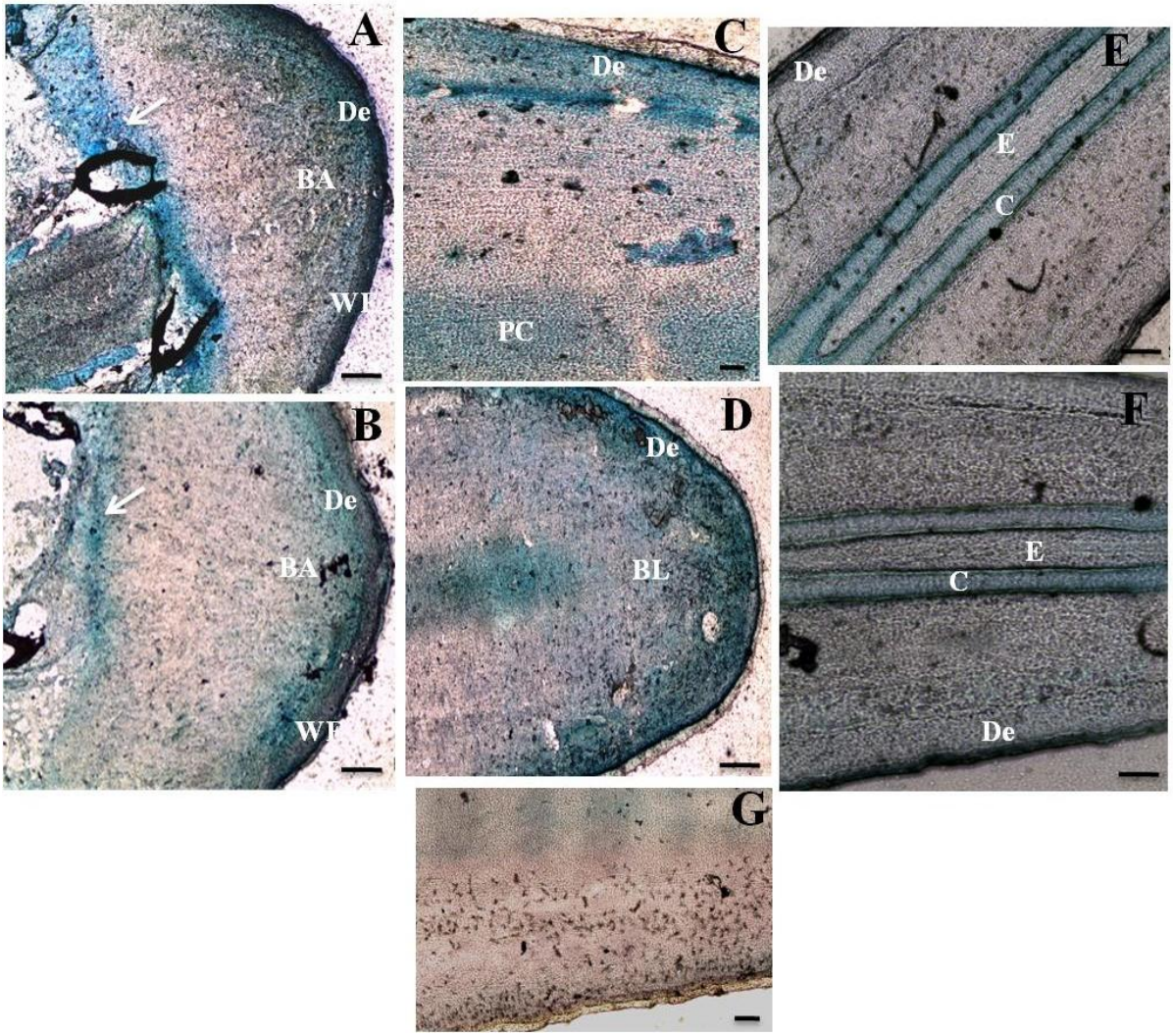



**Figure 2.11. Normal PAGE run under identical conditions as the reverse zymogram. Lanes 1-3 correspond to tail regenerate samples at different stages showing no bands between 20-30kDa. MW corresponds to molecular weight marker.**

**Figure 2.12. Immunohistochemical localization of MMP-2 and MMP-9 in tail regenerates of control *H. flaviviridis* during different stages**

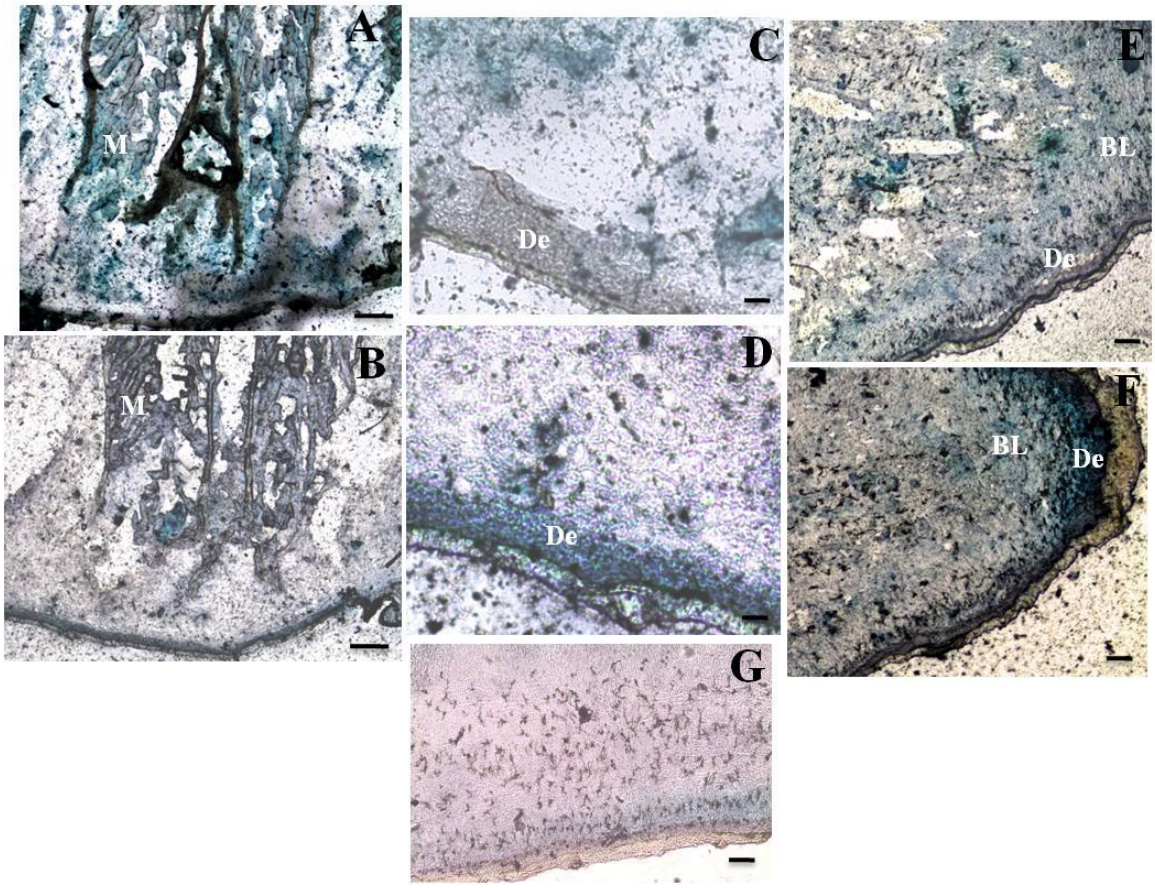



**A, C, E**- MMP-9 localization at wound epithelium (WE), blastema (BL) and differentiation (DF) stages respectively; **B, D, F**- MMP-2 localization at wound epithelium (WE), blastema (BL) and differentiation (DF) stages respectively. **(G)** Negative Control

Main sites of localization include the WE, specially the dermis, blastemal cell accumulation and connective tissue surrounding spinal cord with slight dispersed localization in the entire regenerate at WE stage; dermis, blastemal mesenchyme and procartilage during BL stage and sparse localization in the dermis and cartilage tissue during DF stage. Gelatinases are more intensensely localized during initial stages as compared to late differentiation

BA- cell accumulation prior for blastemal formation; BL- blastema; C- cartilage; De- dermis; E- ependyma; PC- procartilage; WE- wound epithelium; arrows indicate mesenchyme around spinal cord; Scale bars: A, B, D-F = 1mm; C, G = 0.4mm

**Figure 2.13. Immunohistochemical localization of MMP-2 and MMP-9 in tail regenerates of SU5402 treated *H. flaviviridis* during different stages**

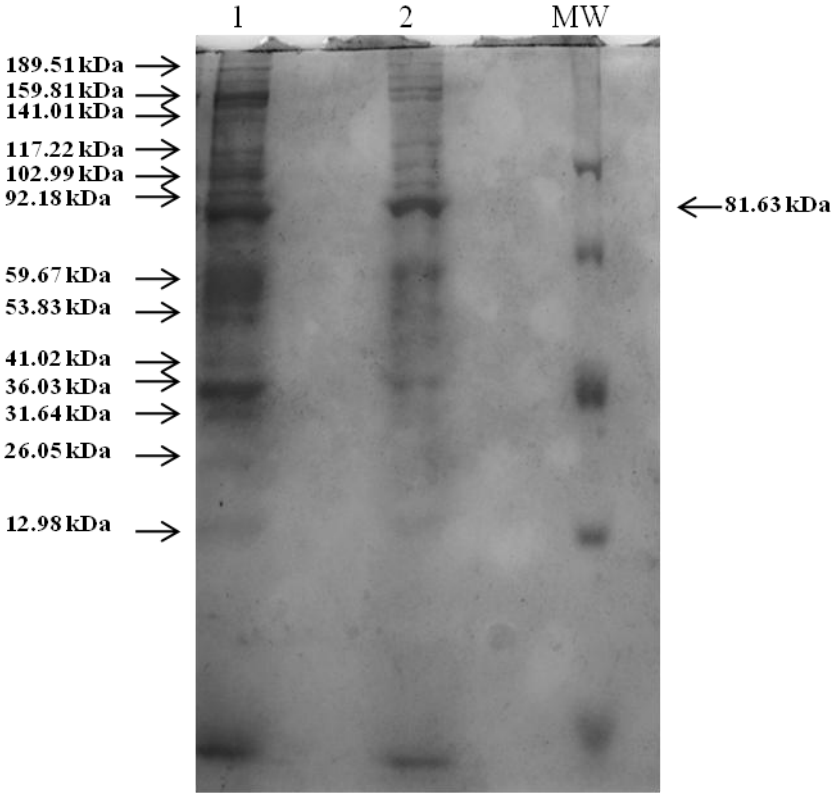



**A, C, E**- MMP-9 localization at 6 dpa (days post amputation), 9 dpa and 13 dpa respectively; **B, D, F**- MMP-2 localization at 6 dpa (days post amputation), 9 dpa and 13 dpa respectively **(G)** Negative Control.

Gelatinase activity visibly affected in treated animals with MMP-2 localized in the dermis during later stages. However, dermis localization is less intense as compared to control group. MMP-9 is seen localized in the muscles during wound epithelium stage.

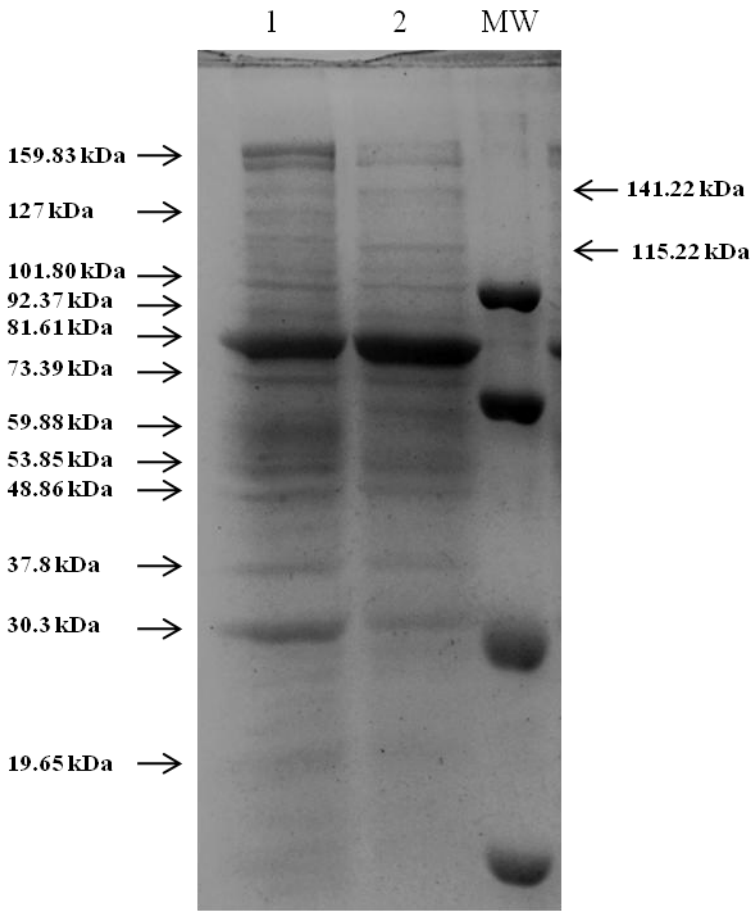
BL- blastema; C- cartilage; De- dermis; M- muscles in stump region; Scale bars: A, B = 1mm; C- G = 0.4mm

**Figure 2.14. SDS- PAGE protein analysis of tail regenerates of control and SU5402 treated *H. flaviviridis* at the wound epithelium stage.**



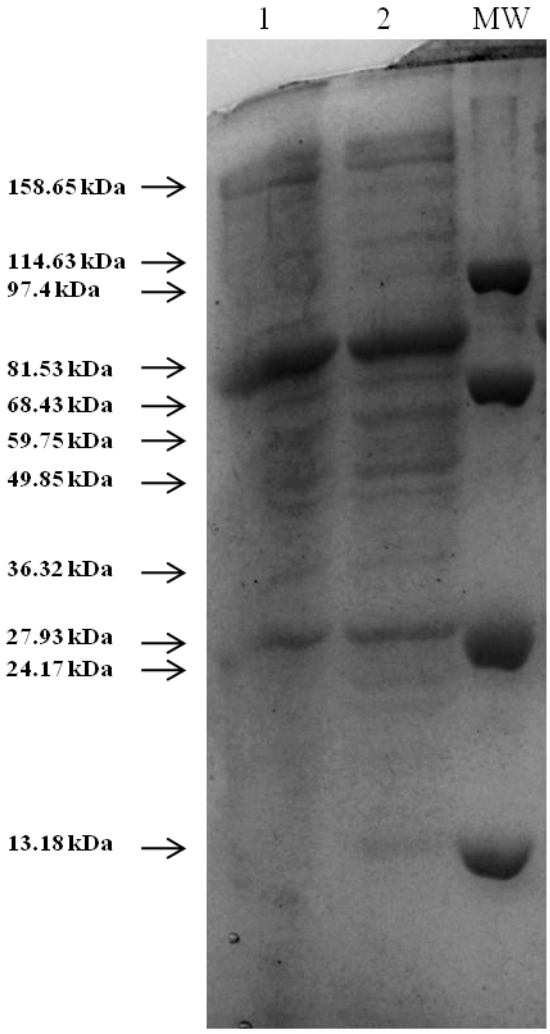

LANE 1 corresponds to control; LANE 2 corresponds to animals treated with FGFR1 inhibitor. MW corresponds to molecular weight marker.

**Figure 2.15. SDS- PAGE protein analysis of tail regenerates of control and SU5402 treated *H. flaviviridis* at the blastema stage.**




LANE 1 corresponds to control; LANE 2 corresponds to animals treated with FGFR1 inhibitor. MW corresponds to molecular weight marker.

**Figure 2.16. SDS- PAGE protein analysis of tail regenerates of control and SU5402 treated *H. flaviviridis* at the differentiation stage.**




LANE 1 corresponds to control; LANE 2 corresponds to animals treated with FGFR1 inhibitor. MW corresponds to molecular weight marker.

# CHAPTER 3

## INFLUENCE OF FGF2 SIGNALLING ON VARIOUS CELLULAR ACTIVITIES DURING TAIL REGENERATION OF *HEMIDACTYLUS FLAVIVIRIDIS*

### INTRODUCTION

Wound healing phase is considered as a key milestone during epimorphosis since, proper completion of which paves way for subsequent restoration of the lost part. Although, rarely defined, a wound is generally acknowledged as all manner of tissue damage resulting in the disruption of the original tissue architecture and homeostasis (Stocum, 1995; Gurtner *et al*., 2008). Wounding is followed by the activation of various extracellular and intracellular events that ultimately act to restore tissue integrity and physiological equilibrium (Gurtner *et al*., 2008). As seen in the previous chapter, inflammatory cascade involving cell migration and matrix reorganization leads to formation of the wound epithelium. Another important process known to be used in response to injury and supposed to play an important role in determining the regenerative ability is apoptosis (Elmore, 2007).

Apoptosis provides a defence mechanism by which damaged and potentially dangerous cells can be eliminated from the wound site. Further, cell-cell and cell-matrix interactions during matrix reorganization are essential in healing and apoptosis might be involved in regulating these interactions. Epimorphosis also requires rapid tissue growth as well as morphogenesis. One mechanism by which cell number is controlled during morphogenesis is by apoptosis (Saunders, 1966; Guha *et al*., 2002). Apoptosis is also known to be involved in sculpting of growing tissue in a number of developmental systems including heart, limb and craniofacial patterning (James, 1994; Graham *et al*., 1996; Nagy *et al*., 1998; Huguet *et al*., 1999; Guha *et al*., 2002; Bastida *et al*., 2004) as well as nervous system pruning (Erdem *et al*., 1998; Honig and Rosenberg, 2000; Bagri *et al*., 2003; Johnston, 2004; Mallat *et al*., 2005). Although little is known about apoptosis in the blastema, a study in planarians, has suggested that apoptosis may be involved in controlling cell numbers, eliminating unnecessary tissues or cells and remodelling the old tissues of regenerating body parts (Hwang *et al*., 2004). Further, studies of *X. laevis* tail regeneration have shown that a degree of apoptosis is an early and obligate component of normal tail regeneration, as early inhibition of apoptosis after amputation abolished regeneration (Tseng *et al*., 2007).

Further, the requirement of apoptosis may also be to balance the large scale proliferative activities occurring in the regenerate. It is known that a period of cell division is a prerequisite for epimorphosis to occur irrespective of which tissues need to be regenerated (Grimes and Goss, 1970; Goss, 1972; Carlson, 1974). Initial wound healing is also dependent on proliferation of epithelial cells to cover the exposed wound surface giving rise to multilayered apical epithelial cap, signals from which are thought to direct subsequent regeneration. Cellular proliferation provides the cell population, the blastema, from which new tissues could be created by differentiation, morphogenesis, and pattern formation (Hay and Fischman, 1961; Iten and Bryant, 1976; Tank and Holder, 1981). Either of the two modes of blastema formation, dedifferentiation or stem cell activation, requires the re-entry of quiescent cells into active cycles of cell proliferation. In general, regenerating tissues are composed of asynchronously cycling cells i.e., not every cell undergoes division at the same time. It is now recognized that blastema cells are typical proliferating cells. They pass through the cell cycle and the important regulatory events occur in the interphase period between mitoses (Mitchison, 1971; Tassava and McCullough, 1978). Further, all systems of epimorphic regeneration show the ability to sustain multiple episodes of cell cycle re-entry with little change in time course a feature possibly contradictory to the fixed proliferative potential of most animal cells (Zilakos *et al*., 1992). Indeed, newt limb blastemal cells can be maintained in culture for more than 200 generations without any signs of crisis or senescence (Ferretti and Brockes, 1988). However, this is not associated with susceptibility but with marked resistance to tumour formation and cells retain ability to undergo differentiation and morphogenesis (Prehn, 1971; Tsonis and Eguchi, 1983).

Another event necessary for the regeneration of the lost tissues is the simultaneous development of new blood vessels in the regenerate. Angiogenesis is a fundamental event that affects physiologic processes (e.g. wound healing, regeneration, the vascularisation of ischemic tissues), and pathologic processes, such as tumour development and metastasis, diabetic retinopathy, and chronic inflammation (Carmeliet, 2005). In tumour cells, angiogenesis is known to be important for cell proliferation and malignant alteration. In fact explosive tumour cell proliferation occurs when the new blood vessels formed by angiogenesis reach the tumour cells (Naumov *et al*., 2006). Similarly, the angiogenesis that occurs during regeneration may be required for supplying nutrients and/or growth factors to blastema cells as well as for supporting their growth and differentiation. Angiogenesis proceeds through extracellular matrix (ECM) degradation, endothelial cell migration, invasion and proliferation and finally reestablishment of cell-cell contacts and lumen formation (Arnold and West, 1991; Folkman and Shing, 1992; Capogrossi and Passaniti, 1997; Giampietri *et al*., 2000).

Angiogenesis is a multifactor process, which is regulated by interplay of a large number of factors. Despite the diversity of factors that participate in angiogenesis, Vascular endothelial growth factor (VEGF) is the most important vascular growth stimulator in adult tissues undergoing physiologic angiogenesis (e.g. proliferating endometrium) as well as angiogenesis occurring in chronic inflammation, wound healing, tumours and diabetic retinopathy (Carmeliet, 2005; Adams and Alitalo, 2007). VEGF is secreted by many mesenchymal and stromal cells (Nagy *et al*., 2007) and induces the migration of endothelial progenitor cells in the bone marrow, and enhances the proliferation and differentiation of these cells at the sites of angiogenesis. In angiogenesis, originating from pre-existing local vessels, VEGF stimulates cells to produce MMPs that degrade the basement membrane and surrounding ECM. As a result, endothelial cells proliferate and migrate towards the interstitium, where they start sprouting. Subsequently pericytes proliferate and migrate towards the newly formed sprouts and induce maturation by forming a single cell layer around the sprout (Bruick and McKnight, 2001; Hoeben *et al*., 2004). Reportedly, VEGF signal inhibition suppresses angiogenesis during zebrafish fin regeneration, but does not completely block regeneration, resulting in formation of avascular tissues lacking clear morphogenesis (Shibuya, 2006). Intriguingly, blastema formation and proliferation are not suppressed. Thus, this study showed that the blood vessels are not indispensable in blastema formation, although they may be needed to support blastema growth and subsequent morphogenesis (Shibuya, 2006).

Involvement of FGF2 in the basic events of regeneration *viz*. wound healing, cell proliferation is well established. It is a known mitogen and affects proliferation of a broad range of cell types both *in vivo* and *in vitro* (Blaes and Allera, 1997; Martin *et al*., 1997; Rusnati et al., 2002) and has been implicated in several types of cancers (Kormann *et al*., 1998). Even cell proliferation during regeneration is dependent upon FGF2 (Poulin *et al*., 1993; Ferretti *et al*., 2001). In addition, one of the mechanisms by which FGF2 regulates cell survival and proliferation is by means of regulating apoptosis (Cooper and Hausman, 2009). Also, FGF2 is considered to have even greater angiogenic potential than VEGF and influences angiogenesis through signalling cascades partially overlapping with those initiated by VEGF (Cross and Claesson-Welsh, 2001). It stimulates endothelial cells to produce both MMPs and VEGF and increases VEGFR expression. It also stimulates endothelial cell migration, pericyte attraction and matrix deposition (Presta *et al*., 2005).

FGF2 evidently influences initial processes of wound healing as well as blastemal proliferative activities during reptilian epimorphosis as can be concluded from the results of the previous chapters. Its localization in several differentiated tissues as well as impaired differentiation of these in FGF2 signal inhibited animals point towards its role in initial differentiation as well, though its influence on later morphogenesis and patterning appears to

be trivial. Hence, it is reasonable to presume that FGF2 might be involved in regulating apoptotic, proliferative as well as angiogenic activities during *H. flaviviridis* tail regeneration contributing to the formation of a proper regenerate. This hypothesis was tested by studying these processes in control and FGF2 signal inhibited lizards. Apoptosis was evaluated through a TUNEL assay as well as localization of caspase-3 in tail regenerates. Proliferative activities were analysed through BrdU incorporation and localization as well as acridine orange staining of the regenerates. Localization of VEGF in the regenerates was done to understand the role of FGF2 in regulating angiogenesis.

Further, an important factor involved in several important events of regeneration appears to be the enzyme Cyclooxygenase-2 (COX-2). COX-2 is a stimulus-inducible enzyme that is implicated in pathological conditions such as inflammation, pain, fever and cancer (Morita, 2002). COX-2 is also an upstream modulator of Prostaglandin $E_2$, one of the inflammatory mediators involved in regeneration. 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). Moreover, a close correlation between the expression of $PGE_2$ and VEGF is being recognized (Gately and Li, 2004; Hitoshi *et al*., 2004; Miura *et al*., 2004). It is known that $PGE_2$ signals are sent and received between the wound epidermis, nerves, fibroblasts, and that they are necessary and sufficient to induce dedifferentiation and the formation of new structures (Murakami and Kudo, 2004; Shen *et al*., 2006). $PGE_2$ is also known to be involved in several processes of the wound healing response (Savla *et al*., 2001; Wilgus *et al*., 2004; Branski *et al*., 2005). High activity of COX and $PGE_2$ are observed during vertebrate appendage regeneration (Appukuttan *et al*., 1993). Recently a study conducted in our lab showed that inhibition of COX-2 induced $PGE_2$ via use of specific COX-2 inhibitor etoricoxib negatively influences tail regeneration in *H. flaviviridis* as well (Sharma and Suresh, 2008, Suresh *et al*., 2009).

Some theories suggest that FGF2 works through COX-2 and prostaglandin pathway (Bikfalvi *et al*., 1997, Foegh and Ramwell, 2004). FGF2 is known to trigger Arachidonic acid metabolism (Bikfalvi *et al*., 1997) which is an important substrate in cyclooxygenase pathways. A ten to 18-fold increase in the expression of COX-2 occurs when stimulated by growth factors, tumour promoters and cytokines (Foegh and Ramwell, 2004). Conversely, there are several reports that cyclooxygenases regulate FGF2 mediated proliferation and angiogenesis (Antoniotti *et al*., 2003; Finetti *et al*., 2008). Thus there appears to be a co-ordinated interplay between FGF2 and COX-2 in regulating events of regeneration. Hence, a localization study of COX-2 in FGF2 inhibited animals and of FGF2 in COX-2 inhibited animals was done during *H. flaviviridis* tail regeneration to elucidate the interaction between FGF2 and COX-2 during this process.

In essence, the current study was designed to assess the role of FGF2 in several important cellular activities occurring during regeneration. FGF2 is now conclusively established as an essential requirement for reptilian tail regeneration (chapter 1) and the knowledge of the way in which it influences these events will provide a broader understanding of its regulation of epimorphosis in a reptilian model.

## MATERIAL AND METHODS

### Animals, Experimental Design and Drug Dosage

The procured animals were acclimatized and maintained in the animal house as described elsewhere (Material and Methods). All experimental procedures were done under hypothermic anaesthesia. On completion of the experimental tenure the lizards were released back to their natural habitat after two months rehabilitation in the animal facility.

First, a study was done for analysing the influence of FGF2 on activities of cell proliferation, angiogenesis, apoptosis and COX-2 activity. A total of 60 lizards of both sexes were selected for this study. The lizards were divided into two groups of thirty each and treated as follows

Group I: This group of animals served as control to the experimental groups and injected with vehicle (1%DMSO).

Group II: The animals were injected with SU5402 (0.7 mg/kg body weight).

Treatment started two days prior to amputation and was continued on every alternate day till the termination of the experiment. Control animals reaching the different stages of regeneration *viz*. wound epithelium (WE), blastema (BL) and differentiation (DF) were selected and the regenerates along with an intact tail segment were collected by inducing autotomy. Regenerates of treated animals were collected on the day when the control animals reached the appropriate stages irrespective of whether treated animals attained the same stage or not. For COX-2 localization and apoptotic studies, regenerates were also collected at 48h post amputation (hpa) from both the groups. Regenerates were processed further for the localizations.

A further study to understand COX-2 regulation of FGF2 in the regenerative process was also carried out. A total of 24 animals were used in this study and were given the following treatments.

Group I: This group of animals served as control to the experimental groups and injected with Tris buffer (vehicle).

Group II: The animals were injected with specific COX-2 inhibitor etoricoxib (50mg/kg body weight).

Etoricoxib is soluble in alkaline pH and was prepared in Tris Buffer (pH 8.8) fresh before use. The procedures for amputation and treatment remained the same as for the first study.

Regenerates were collected from animals of both groups at the various defined stages and processed for FGF2 localization.

**Immunofluorescent localization of FGF2, COX-2, VEGF, Caspase-3**

Briefly, regenerates were embedded in Tissue-Tek OCT and frozen at -20ºC. For immunolabelling, longitudinal cryosections (8-10µm) were fixed in acetone and air dried for 15 minutes. Sections were then rehydrated with PBST followed by blocking with normal serum for 1-2 hours at room temperature (RT). Sections were then incubated with appropriate primary antibody (appropriate dilutions of Anti-FGF2, Anti-COX-2, Anti-VEGF or Anti-Caspase-3 antibody) overnight inside a moist chamber at 4ºC. Following day, sections were washed with PBST thrice and incubated with a corresponding FITC conjugated secondary antibody for 2 hours at RT. Sections were then washed with PBS and mounted in 1:1 mixture of PBS:glycerol and observed under a fluorescent microscope (Leica DM2500). Same procedure was followed for negative control sections except that these were incubated with PBS-BSA instead of the primary antibody. Mammalian antibodies have been used for these localizations considering the evolutionary conservation of these proteins (Nugent and Iozzo, 2000; Grosser *et al*., 2002; Holmes and Zachary, 2005; Sakamaki and Satou, 2009).

***In vivo* BrdU incorporation and immunolocalization**

Intraperitoneal injection of BrdU (100mg/kg body weight) was given at different stages of regeneration and the regenerates were collected after 24 hours. Tissues were embedded in OCT and fresh frozen sections (8-10µm) were taken on poly-l-lysine coated slides. The sections were fixed in cold acetone and air dried for 15 minutes followed by treatment with 2N HCl for 30-60 minutes at 37$^{o}$C. Sections were then rinsed in borate buffer for 10 minutes and then rehydrated in PBS at RT. Sections were blocked using normal serum for 1-2 hours at RT, and incubated with primary antibody (1:100 dilution of Mouse Anti-BrdU) overnight inside a moist chamber at 4ºC. Next day, sections were washed with PBS and incubated with FITC conjugated secondary antibody (1:50 dilution of Goat Anti-Mouse IgG-FITC) for 2 hours at RT, washed, mounted with PBS:glycerol (1:1) and observed using a fluorescent microscope (Leica DM2500 utilizing LAS EZ software).

**Histofluorescent localization of nucleic acids**

Regenerates were excised and immediately transferred to a cryostat microtome maintained at -20ºC. After embedding in OCT, longitudinal cryosections (8µm) of these were taken on clean glass slides. Sections were immediately fixed in ice-cold acetone for 1-2 minutes, kept in phosphate buffer (pH 6.0) for 2-3 minutes and stained with a 1:10 dilution of Acridine Orange stock solution for 1-2 minutes. The sections were then observed using a fluorescent microscope (Leica DM2500) with epi-illumination filter settings of 440nm excitation filter and 510 nm barrier filter.

**TUNEL staining for apoptotic analysis**

Apoptosis was detected in the regenerates using a TUNEL kit (Gen Script, USA) Briefly, tissues were excised, frozen at -20ºC and embedded in OCT. Longitudinal cryosections (8-10µm) were taken on clean glass slides and fixed with cold acetone for 1-2 minutes and proceeded for TUNEL staining. Concisely, biotinylated nucleotide is labelled at the DNA 3'OH ends using the natural or recombinant terminal deoxynucleotidyl transferase (TdT). Then, HRP labelled streptavidin is bound to these biotinylated nucleotides, which are detected using the peroxidise substrate, hydrogen peroxide, and 3, 3'-diamniobenzidine (DAB), a stable chromogen. Using this procedure, apoptotic nuclei are stained dark brown.
(For details on the techniques used, please refer the section Material and Methods)

**RESULTS**

**Apoptotic activity during tail regeneration**

Since FGF2 is known to influence tail regeneration in *H. flaviviridis* (chapter 1), its role in the regulation of apoptotic activities during this process was evaluated. In control animals, apoptotic cells were prominent during 48 hpa and also during the formation of the wound epithelium. Maximum evidence for apoptosis was observed in the areas of spinal cord and muscle bundles of the intact tail segment. Cell death was also evident in the region of the wound epithelium and the epithelium of the intact tail segment. Blastemal and differentiation stages were characterized by localized areas of cell death along the epithelium, particularly the dermal region. Blastemal mesenchyme showed sparse apoptosis. Newly developing muscle bundles and ependyma of the regenerate also showed the presence of apoptotic cells. This indicates that apoptosis might be required for the patterning of these differentiating tissues during later stages. However, during these stages also an appreciable amount of cell death was observed in the epithelial and muscular areas of the intact tail. Overall, significant apoptosis was observed in the early stages as compared to later growth stages (Figure 3.1A-G).

In the SU5402 treated lizards, FGF2 signal inhibition led to a large scale cell death during the early stages of regeneration *viz*., 48hpa and 6days post amputation (dpa). The degree of apoptosis was evidently higher as compared to control animals, but the pattern remained the same with maximum apoptosis in the muscle bundles and nervous tissue and even in and around the epithelium in the SU5402 treated lizards. Cell death was not only restricted to this initial period but continued even after 8 and 11dpa, whereas the control animals during this stage showed comparatively negligible apoptotic activity. Intensity of cell death around the wound epithelium, blastemal mesenchyme, regions of nervous and muscular tissue was higher during these stages as compared to control animals (Figure 3.2A-G). Negative control sections were stained positively only in the outer keratinized part of the epithelium of the

intact tail region. These points were kept in mind while interpreting the results (Figures 3.1H-J, 3.2H, I).

Further, to elucidate the apoptotic process and to consolidate the results of the TUNEL study, activity of one of the executioner caspases, caspase-3, a standard marker of apoptosis was also evaluated in the control and treatment groups. Results apparently are in accordance to the above observations. As already observed, in the SU5402 treated animals, caspase-3 localization was predominantly seen in the regions of the muscle bundles, spinal cord and epithelium in the region of the intact tail segment. Intense staining of wound epithelium and weak staining of blastemal accumulation was also evident on completion of wound healing (Figure 3.4A-G). Control animals showed comparatively regulated activity of the caspase-3 in these regions during initial stages. A good amount of staining could be observed in the promuscle aggregates and developing ependymal tube during later stages (Figure 3.3A-F). Also the keratinized epithelium was positively labelled in contrast to the negative control sections. Conclusive results could not be made from the TUNEL staining because of the nonspecific staining observed in the negative control sections in this region, but caspase-3 localization proved otherwise (Figures 3.3G-I, 3.4H, I).

To sum it up, higher levels of cell death in the regenerate during early stages in SU5402 treated animals cannot be neglected, evidently pointing towards a protective role of FGF2 during reptilian tail regeneration.

**Cell proliferation during regeneration**

Initial wound healing and blastema formation are now known to get delayed in SU5402 treated animals (chapter 1), suggestive of the role of FGF2 in cellular proliferation. Hence, BrdU incorporation and subsequent immunolocalization of BrdU labelled cells was done to assess the level of proliferative activity in control and FGF2 signal inhibited animals. Formation of the wound epithelium was accompanied by proliferative cells in the region near the new epithelium and also in the mesenchymal zone beneath it. Labelled nuclei became more prominent during blastema and differentiation stages of regeneration. Proliferating zones included the regions of promuscle aggregates, the blastemal zone, connective tissue that lies between the muscle and nervous tissue and the dermal region. Among the labelled cells, regenerating cells of the ependyma were also prominently seen during late differentiation. However, in the treatment group regenerates, number of cells entering cell cycle was markedly affected. An appreciable number of labelled nuclei were only seen during later stages i.e. after 9-11 dpa in the treatment group. Similar areas were labelled for the actively dividing cells like the mesenchyme and the epithelial region, only the level of proliferation in these regions was greatly affected. Moreover, during blastema and differentiation stages, labelled cells were significantly confined to the region that lies at the

junction of the amputation surface and the newly regenerating tail, suggestive of the source of dividing cells. In the entire regenerate this region exhibited a significant number of dividing cells. In the treatment group the only region where appreciable numbers of labelled cells were observed was in this region however, the number of labelled cells was fewer compared to the controls (Figures 3.5, 3.6).

In support of the above cell proliferation study, histofluorescence localization of nucleic acids was done through acridine orange staining of the regenerates at proliferative stages. The level of DNA and RNA fluorescence can be an indicator of the replicative, transcriptional and translational activities during regeneration. It could be observed from the results, that the fluorescent signals of both the nucleic acids were affected by SU5402 treatment. Following wound healing, yellow-green fluorescence could be very well observed in the entire regenerate, with sparse red fluorescence near the epithelium. With subsequent proliferation and blastema formation, RNA localization was much prominently seen in and around the epithelial area. DNA signal was pronounced during this stage and could be seen in the entire matrix of the regenerate, with regions beneath the dermis, developing muscle bundles and ependymal region prominently emitting yellow-green fluorescence. During differentiation, observed RNA localization increased to its maximum amongst the several stages studied along with equally good fluorescence of DNA. It was of particular interest that a significant localization of DNA again in the region that lies at the junction of the intact tail and the regenerate was observed (Figure 3.7). In the treatment group, the intact tail segment as well as the junction of the stump and regenerate had the maximum fluorescence of DNA during the entire experiment. However, the regenerating area definitely showed comparatively less signal as compared to control animals, but the pattern remained the same with RNA signal around the epithelium. After about 10dpa, an appreciable AO staining of the DNA and RNA in the regenerate could be observed in this group (Figure 3.8).

Thus evidently, amount of DNA and RNA localized during regeneration can be correlated to a certain extent with the activity of cellular proliferation between the two groups. Affected nucleic acid levels in the treatment group can be considered to be in accordance with the BrdU localization results suggesting that the replicative and later transcriptional activities might be getting affected by inhibited FGF2 signalling.

**VEGF localization during regeneration**

Successful proliferation of the regenerate is always accompanied by angiogenesis. Since, FGF2 inhibition altered the initial proliferative activities of regeneration (results cited above), its effect on angiogenic activity too was studied through immunolocalization of the angiogenic molecule VEGF.

VEGF was found to be actively localized during all defined stages of regeneration in the control animals. Labelling was evident in all the major hallmark structures of each stage. The major site of localization at the completion of wound healing was the newly developed epithelium, which was intensely stained. Labelling was also evident among the migrating cells that later might get incorporated into the blastema. Subsequent blastema stage had much better localization of VEGF at this mesenchymal region beneath the epidermis. Differentiation stage had positively labelled ependyma and regenerating muscle bundles. In comparison to the early wound healing stage, labelling was much better comparatively in the blastemal and differentiation stages. The intense positive staining of the muscle bundles of the intact tail region during the entire period of experiment was noticeable. The keratinized epithelium of this region was also positively stained (Figure 3.9A-F).

For the treatment group, labelling was not as intense as the control animals. VEGF activity definitely seemed to get affected by ablation of FGF2 signal as could be observed from the poor labelling of the AEC which was intensely labelled for the control group. The mesenchymal cells were also poorly labelled. Lesser intense labelling of the muscle bundles and keratinized epithelium of the intact tail region suggested that VEGF activity in this region too was affected by FGF2 signal inhibition. Much better labelling was observed in the intact spinal cord of the treated lizards. However, VEGF angiogenic activity definitely seemed to be diminished by FGF2 signal inhibition (Figure 3.9G-K). As the treatment group never attained certain histological structures during the period of experimentation, results could be debatable, but SU5402 affected VEGF localization in the intact tail segment also, proving beyond doubt that FGF2 signalling is required for proper VEGF activity during regeneration.

**FGF2 and COX-2 interaction during regeneration**

After 48hpa, significant COX-2 localization was observed in the epithelial region with both the keratinized and dermal parts positively stained. Prominent localization was also seen in the muscle bundles of this region. During the later stage when the WE is formed, localization was still evident in the dermal part of the epithelium and the AEC as a whole was positively stained. During later stages, COX-2 localization became prominent in the developing muscle bundles as well and connective tissue around it. Localization was less prominent during subsequent differentiation stage in the epidermal region although, labelling in the muscle bundles and blood vessels could still be detected (Figure 3.10).

Surprisingly, COX-2 localization was not markedly affected by FGF2 signal inhibition, as the SU5402 treated lizards also had comparative COX-2 activity following amputation in the dermal region of the epithelium. The epidermal region also showed comparable fluorescence. Once the wound epithelium was formed, it was also found to be positively stained. However, the muscle bundles of intact tail segment were weakly labelled as compared to the control

animals. As the treated animals did not reach a similar histoarchitectural state as the control animals, localization differences between the two experimental groups is a matter of dispute, but localization post 48h of amputation definitely point towards COX-2 localization being affected in the muscular region although the inflammatory region has comparable activity of this enzyme even in the treatment group (Figure 3.11).

Since COX-2 activity did not significantly seem to get affected by FGF-2 signal inhibition, and considering the role of COX-2 induced FGF2 pathways in physiological regulation as reported by some studies, an experiment to examine whether COX-2 regulates FGF2 at some level during the regenerative process was investigated. This was done through inhibition of COX-2 with specific COX-2 inhibitor etoricoxib and the expression levels of FGF2 were studied in the animals. From the results it was evident beyond doubt that COX-2 inhibition appreciably altered the FGF2 localization in the regenerating tissues. Irrespective of the stage of regeneration, FGF2 labelling was weaker at each stage in the treated animals as compared to the control group. The significantly intense FGF2 labelling in the developing epithelium, the blastemal mesenchyme and the differentiating tissues in the control animals as compared to the weak labelling that was observed for the treatment group (Figures 3.12, 3.13).

Thus it is very much possible that FGF2 signal inhibition does not largely influence the inflammation mediated via COX-2 although it does affect its localization in the muscle tissues, but COX-2 definitely does seem to play a role in regulating FGF2 signalling during reptilian regeneration process. It is possible that this inflammatory mediator of wound healing must be one of the inducing factors for the release of the neurotrophic FGF2 for subsequent proliferation and in its absence the quality of regeneration may be compromised.

**DISCUSSION**

Regeneration is a complex process possessing a number of distinct phases (Gardiner *et al*., 2002). Several cardinal events like programmed cell death, controlled cell proliferation and angiogenesis form the basis of this extraordinary ability to restore the lost tissues. Participation of several countless factors and their regulated interplay is absolutely necessary to take care of such events that ultimately lead to proper completion of regeneration.

FGF2 is one such factor which is deemed necessary in the regenerative ability of several animal models. In addition, from the previous experiments, FGF2 can be conclusively said to be essential for tail regeneration in the lizard model, *H. flaviviridis* as well (chapter 1 and 2). Hence, to further understand the role played by FGF2 in this process, it was thought worth evaluating its importance in regulating some of the key cellular events of epimorphosis *viz*. apoptosis, cellular proliferation and angiogenesis.

A certain degree of cell death by apoptosis is an important feature of any morphogenetic process, be it development or regeneration (Bastida *et al*., 2004; Hwang *et al*., 2004; Mallat *et al*., 2005; Tseng *et al*., 2007). Apoptosis is not only required to clear the damaged cells but this regulated cell death may also be essential for balancing the process of extensive cellular proliferation and useful for tissue patterning which are characteristic of any morphogenetic event. In fact, an endogenous early apoptotic event is reported to be required for regeneration despite the massive tissue proliferation involved (Tseng *et al*., 2007). The existence of apoptosis has been reported in the context of regeneration in planaria (Hwang *et al*., 2004), in *Xenopus* (Suzuki *et al*., 2005) and in newts (Kaneko *et al*., 1999). Apoptosis inhibition in the early phases post amputation not only abolished regeneration but also affected proliferation and neuronal patterning of *Xenopus* tail regeneration buds. Moreover, refractory stage tail amputation resulted in an increase in the apoptotic population in the bud as compared with regenerative stages, suggesting that a tight control of a specific level of apoptosis is required for proper regeneration (Tseng *et al*., 2007). Whether FGF2 is a factor that mediates control of such an event during *H. flaviviridis* tail regeneration is not known. Since, FGF2 inhibition delayed the regenerative event and also affected differentiation of several tissues, its role if any, in regulating the apoptotic event during reptilian epimorphosis was evaluated.

FGF2 is a known mitogen and one of the mechanisms by which it augments cell proliferation might be the inhibition of apoptosis. From combined results of TUNEL staining and Caspase-3 localization in tail regenerates of control and SU5402 treated lizards, it could be observed that FGF2 signal inhibition certainly leads to unregulated apoptosis of cells during regeneration. A greater degree of apoptosis was observed in the early stages after amputation and this decreased as the regeneration progressed with an appreciable but not intense level of apoptosis again seen at the differentiation phase in the control group. Compared to control animals, SU5402 treated animals showed an elevated level of cell apoptosis during all stages of experiment. Nevertheless, sites of cell death remained the same in both the groups which included the spinal cord, muscle tissue as well as region near the epithelium. Higher levels of apoptosis at all these sites were observed for the treated group. It is possible that FGF2 signal inhibition might have led to this increased cell death, since FGF2 is known to be a cell survival factor and greater apoptotic activity in this group could be attributed for the delayed regeneration that in turn might have delayed the morphogenesis causing poor histoarchitecture (refer chapter 3 for histology). There are several reports that FGF2 is involved in regulating cell survival by inhibiting apoptosis in a wide variety of cells. FGF2 inhibits TNF-α mediated apoptosis in the murine chondrocytic ATDC5 cell line through upregulation of Bcl2-A1 and Bcl-xL. It also inhibits apoptosis in retinal pigmented epithelial cells involving an ERK2 dependent Bcl-x production (Bryckaert *et al*., 1999) This suggests that the Bcl2 family proteins are targets of FGF2 signalling in mediating cell survival by regulating apoptosis (Kim *et al*., 2012). Fuks *et al*. (1994) demonstrated that the FGF2

administration in mice before and after irradiation inhibited apoptosis in endothelial cells and protected against lethal radiation pneumonitis. The protein ccp1, a downstream target of FGF2 signalling is known to regulate cell proliferation and apoptosis in neuroblastoma cells (Pellicano *et al*., 2010). FGF2 mediated *survivin* expression inhibits apoptosis through direct interaction with caspase-3 in small cell lung cancer cells (Xiao *et al*., 2008). In contrast, FGF2 is also known to induce apoptosis in certain cells like rat myofibroblasts (Funato *et al*., 1997), neural retinal cells in developing chicks (Yokoyama *et al*., 1997), human breast cancer cells (Wang *et al*., 1998) and in differentiating osteoblasts (Mansukhani *et al*., 2000). FGF2 is also known to differentially regulate members of the TNF superfamily of death domain receptors and their ligands. On one hand, FGF2 inhibits Fas death pathway and on the other, it can also induce apoptosis by activation of TNF-α pathway in neuronal cells (Eves *et al*., 2001). Nevertheless, these findings are indicative of the role of FGF2 in regulating cell death and survival.

Considering the fact that FGF was discovered as a mitogen promoting proliferation of fibroblasts (Gospodarowicz *et al*., 1986), the proliferative potential of FGF2 comes as no surprise. An increasing body of evidence shows that FGF2 produced by autosecretion or parasecretion promotes cell proliferation and inhibits apoptosis (Song *et al*., 2000; Sekimura *et al*., 2004). As seen previously, FGF2 signalling is essential for blastemal proliferation and subsequent tail outgrowth as both these processes were hampered in SU5402 treated lizards. Presumably FGF2 influences the rate of cell proliferation between the two groups. This hypothesis was tested by *in vivo* BrdU incorporation and subsequent localization of BrdU labelled cells. Results showed a lower cell proliferation rate in FGF2 signal inhibited animals during all the proliferative stages of regeneration studied. Evidently, FGF2 confers a proliferative potential to the blastemal cells during reptilian regeneration. This is in accordance with regeneration studies in other animal models such as newts and zebrafish, reporting a similar mitogenic influence of FGF2 on blastemal cells and causing regulated cell proliferation. During amphibian tail regeneration, FGF2 has been shown to increase the proliferation of cells and accelerate the regeneration process (Poulin *et al*., 1993; Hata *et al*., 1998; Ferretti *et al*., 2001). It has also been suggested that during urodele limb regeneration, FGF2 produced in the limb mesenchyme could act as an autocrine factor stimulating blastemal cell proliferation and as a paracrine factor promoting in the permissive epidermis (AEC) the synthesis of several forms of FGF that in turn promote blastema growth and pattern formation (Stoick-Cooper *et al*., 2007).

The proliferative role of FGF2 might be due to its direct effect on the synthesis of DNA, which is needed by rapidly dividing cells of the regenerate. In fact FGF2 is known to be an active participant during cell cycle (Korr *et al*., 1992; Liu *et al*., 1997) and induces a single re-entry of G0 rat astroglial cells into the mitotic cycle. Zeitler *et al*. (1997) have reported that

FGF2 induces cell cycle progression from G0/G1 to S phase in endothelial cells as well. Regulation of proliferation is also essential and FGFs provide this by controlling the length of the G1 phase. Addition of FGF2 in cortical neuron culture derived from mouse at E14-E16 showed shortening of the G1 length and increase in proliferative divisions, indicating that FGF2 controls cell proliferation via its control of G1 length (Lukaszewicz *et al*., 2002).

Besides, FGF2 is known to be mitogenic for many different cell types both *in vitro* and *in vivo*. FGF2 promotes *in vitro* proliferation of human bone marrow stromal cells (Martin *et al*., 1997), bovine and human corneal endothelial cells (Hoppenrejis *et al*., 1994) and neural precursors isolated form adult rat brain (Richards *et al*., 1992). FGF2 has also been implicated in several cancers. FGF2 and FGFR1 and 2 have been shown to be involved in prostatic (Giri *et al*., 1999) and pancreatic (Kormann *et al*., 1988) cancer in humans. Inhibition of either FGF2 or FGFR1 reduced ERK1/2 activation, cell proliferation, and survival in uveal melanoma cells (Lefevre *et al*., 2009). Furthermore, the proliferation and differentiation of bovine osteoblasts are stimulated by FGF2 (Globus *et al*., 1988) while it is an exogenous regulator of smooth muscle cell migration and proliferation in humans (Blaes and Allera, 1997). Likewise the proliferation and differentiation of normal human melanocytes, mesodermal tissues, such as fibroblasts and endothelial cells, as well as neuroectodermal cells in humans are dependent on FGF2 (Halaban *et al*., 1992; Bennett and Schultz, 1993; Bhora *et al*., 1995; Gibran *et al*., 1994). Contrary to this, FGF2 inhibits proliferation of rat chondrosarcoma cells and arrests cell cycle at G1 phase (Aikawa *et al*., 2001). Thus FGF2 promotes cell proliferation in some cell types, while inhibits in others. Nuclear translocation of FGF2 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).

Since FGF2 conclusively plays a role in influencing rate of proliferation during reptilian regeneration, further, in support of this result, acridine orange staining of the nucleic acids in the regenerates was done. Qualitative differences in the levels of nucleic acids between control and treatment group is very much evident from the results obtained. Control group apparently had better DNA and RNA fluorescence in the regenerate during various proliferative stages as compared to SU5402 treated lizards which showed good fluorescence only after 12-13 days post amputation. The level of nucleic acids though not indicative of proliferative activities but can be correlated to it, given that an active process like proliferation occurring during regeneration can be related to processes of DNA replication and subsequent transcription and translation of several proteins to regulate such a process and meet the demands of the rapidly dividing cells and considering the results obtained, FGF2 definitely seems to be involved in these molecular processes related to proliferation.

Another important thing observed from the proliferation studies was that a significant number of dividing cells are present at the junction of the stump and the regenerate during all the stages of regeneration studied. This region also showed intense nucleic acid staining with acridine orange. This region seems to provide a continuous input of cells into the regenerate and hence proliferative activities of resident stem cells in this mesenchymal zone may be significantly responsible for subsequent blastema formation and restoration of the lost tail. Nevertheless, it is important to note that proliferative activities in this region (both BrdU labelled cells and nucleic acid localization), are also significantly affected by FGF2 signal inhibition.

Angiogenesis at the site of injury is another important process that paves way for proper regeneration. The process is tightly regulated by several extracellular signals with one of the most relevant agents displaying angiogenic properties, and the first to be identified, as FGF2 (Bikfalvi *et al*., 1997). Since, SU5402 impaired growth rate as well as quality of the regenerate formed (chapter 1), it was worth evaluating the regulation of angiogenic process by FGF2 during *H. flaviviridis* tail regeneration. This was done through VEGF localization in the regenerates of control and SU5402 treated lizards. VEGF is another important angiogenic regulator and its signalling stimulates the survival of endothelial cells, their proliferation and their motility, initiating the sprouting of new capillaries (Bruick and McKnight, 2001). Results of the current study showed that activity of VEGF is definitely downregulated by FGF2 signal inhibition. Localization of this molecule in several tissues of the regenerate was comparatively less intense in the FGF2 signal inhibited animals. VEGF inhibition is known to affect morphogenesis and tissue patterning, but does not abolish regeneration (Shibuya, 2006). Hence, it is possible that the poor histoarchitectural features observed in the SU5402 treated animals (chapter 1) is induced by VEGF downregulation due to FGF2 signal inhibition. Differences in VEGF activity in control and treated animals being very much evident, it can be concluded that FGF2 is definitely required for VEGF mediated angiogenesis during reptilian regeneration. Moreover, FGF2 being a potent angiogenic molecule, its inhibition in itself is a limiting factor for the formation of a proper regenerate.

Besides, there are several reports suggesting that FGF2 regulates VEGF activity and angiogenesis. It induces VEGF expression in the endothelial cells of forming capillaries through autocrine and paracrine mechanisms (Seghezzi *et al*., 1998) and causes neovascularisation indirectly by activation of VEGF/VEGFR system (Presta *et al*., 2005). Endothelial cell tube formation stimulated by VEGF in murine embryonic explants, depends on endogenous FGF2 (Tomanek *et al*., 2001). Similarly, FGF2 antibodies have also been shown to block VEGF-induced angiogenesis *in vitro* (Mandriota and Pepper, 1997). Adipose stem cells are known to produce angiogenic factors, including VEGF that inhibit hyaline cartilage regeneration and FGF2 is known to eliminate this apoptotic effect of VEGF (Lee *et*

*al*., 2012) suggesting FGF2 regulation of VEGF activity. Proliferation and differentiation of VEGFR-$2^+$ hemangioblast precursors from the mesoderm is promoted by FGF2 (Poole *et al*., 2001). In embryoid bodies, in which VEGF/VEGFR function is impaired, FGF2 stimulates the formation of endothelial cell clusters that fail to develop into primitive vessels (Magnusson *et al*., 2004). FGF2 can also induce vascular permeability directly and indirectly by upregulating VEGF and proteases and this has a role during inflammatory phase of wound healing (Reuss *et al*., 2003). FGF2 and VEGF are the most potent angiogenesis inducers and have a synergestic effect on angiogenesis (Pepper *et al*., 1992; Asahara *et al*., 1995) and hence, often simultaneous targeting of both is implicated in controlling tumour growth and metastasis (Alessi *et al*., 2009). Considering their synergistic interaction, FGF2 may require the activation of the VEGF/FGFR system for promoting angiogenesis. Conversely, VEGF may require FGF2 in order to exert its angiogenic potential under defined experimental conditions. In this respect, FGF2 and VEGF appear to show a combined effect on the maturation of blood vessels in different experimental settings (Nillesen *et al*., 2007; Rophael *et al*., 2007).

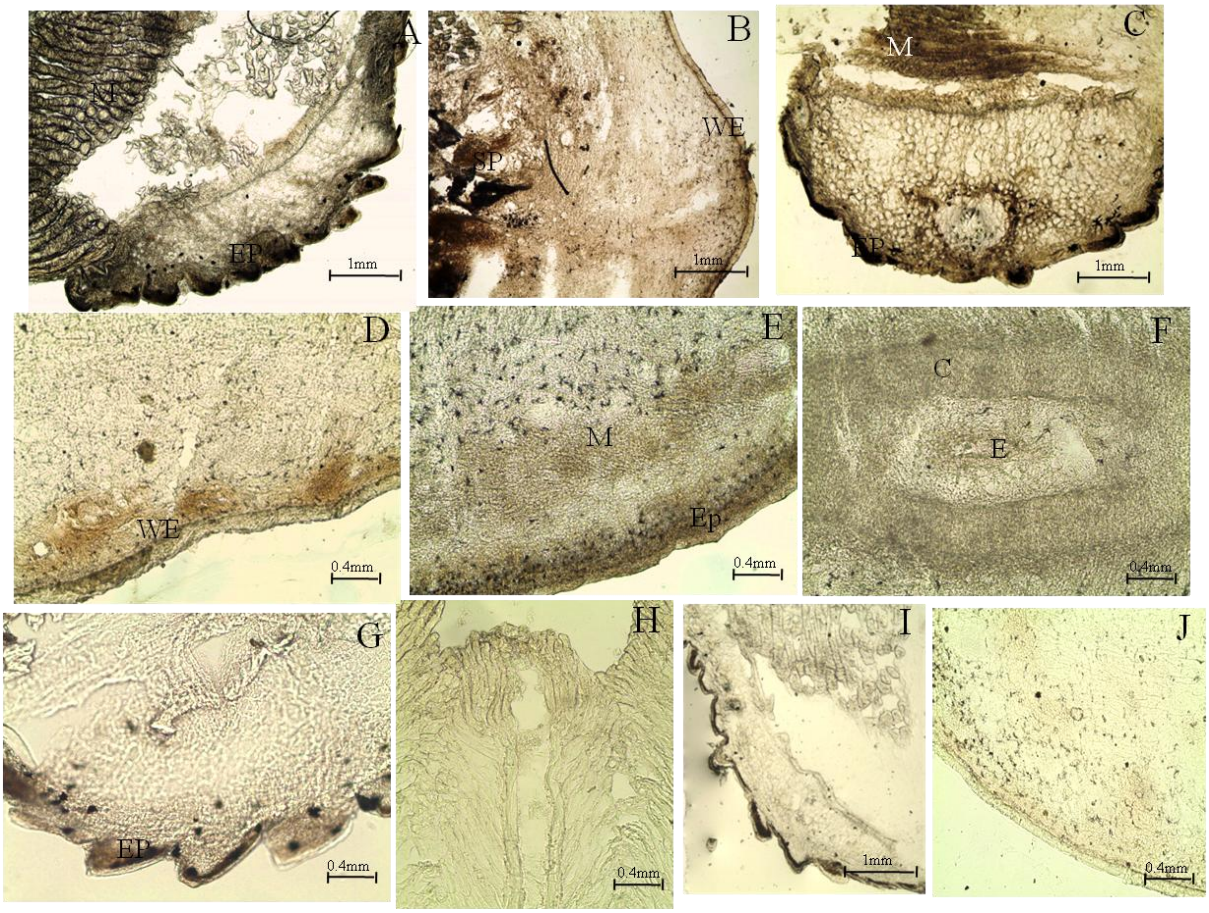
In addition to FGF2, another important inducer of angiogenesis is the cyclooxygenase enzyme COX-2. COX-2 is an inflammatory mediator that is involved in wound healing and tissue repair besides angiogenesis (Kurie and Dubois, 2001; Murakami and Kudo, 2004; Wilgus *et al*., 2004; Shen *et al*., 2006). There are several reports showing a direct association between COX-2 and FGF2 mediated signalling and these reports suggest a two-way regulation of this interaction. FGF2 induces prostaglandin synthesis in a variety of cell types, often in the context of angiogenesis or tissue healing (Kawaguchi *et al*., 1995; Kage *et al*., 1999; Hosono *et al*., 2011) and stimulates $PGE_2$ synthesis in intestinal epithelial cell line via p38α-dependent (p38 MAPK) increase in COX-2 mRNA stability (Tessner *et al*., 2003). FGF2 administration in retinal epithelial cells is known to cause an increase in COX-2 mRNA (Ershov and Bazan, 1999). COX-2 and hypoxia regulated proteins are modulated by FGF2 in acute renal failure (Villanueva *et al*., 2012). FGF2 accelerates astrocyte migration in a scratch model *in vitro* which is a key factor in the repair mechanisms orchestrated by FGF2 and this effect is partly mediated by an upregulation of COX-2 (Lichtenstein *et al*., 2012). Conversely, COX-2 inhibition suppresses FGF2 expression in human oesophageal adenocarcinoma (Baguma-Nibasheka *et al*., 2007). Inhibition of COX-2 by celecoxib delays tumour growth and metastasis in xenograft tumour models as well as suppresses FGF2 induced neovascularizaton of the rodent cornea indicating that FGF2 activity depends upon COX-2. Moreover, COX-2 inhibition also reduces proliferation and induces apoptosis possibly via inhibition of FGF2 (Leahy *et al*., 2002). $PGE_2$, a downstream product of COX-2 regulates angiogenesis via activation of FGFR1 (Finetti *et al*., 2008). Over expression of COX-2 which characterizes many epithelial tumours as well as their endothelial population, is accompanied by enhanced expression and production of angiogenic factors like VEGF and

FGF2 (Abdelrahim and Safe, 2005; Larkins *et al*., 2006). $PGE_2$ upon binding to its membrane receptor activates a signal cascade that through a complex array of intermediate steps leads to the extracellular ligands stimulating growth factor receptors and producing tumour growth (Gschwind *et al*., 2001). Moreover, FGF2 and VEGF mediated angiogenesis is partly dependent on increasing expression of COX and $PGE_2$ production (Hernandez *et al*., 2001; Salcedo *et al*., 2003).

The association of COX-2 and FGF2 signalling pathways is very much evident from these reports. Considering this fact, whether an interplay in regulation of these factors exists during *H. flaviviridis* tail regeneration was evaluated by a two way study. Influence of FGF2 inhibition on COX-2 activity and vice versa was analyzed by a localization study of COX-2 and FGF2 in the tail regenerates respectively. Results show that SU5402 did not substantially affect COX-2 activity during regeneration when compared to control group. However, its localization in muscle region definitely seems to get affected. Conversely, COX-2 inhibition by etoricoxib was found to significantly affect FGF2 localization during regeneration. Obvious differences in staining intensity could be observed between the control and treatment groups in several areas of the regenerate. These results suggest that FGF2 may not largely influence COX-2 mediated signalling during regeneration of the studied animal model. However, COX-2 activity evidently seems to be necessary for FGF2 expression and subsequent signalling. As such there are reports that COX-2 induced $PGE_2$ is an important regulator in the processes of wound healing, proliferation, myogenesis and angiogenesis in the same animal model (Suresh *et al*., 2009), and its inhibition leads to improper and delayed regeneration (Sharma and Suresh, 2008). Evidently it also must be regulating FGF2, which itself is a prerequisite for a successful regenerate formation.

Conclusively, from this study, FGF2 can be regarded as a factor essential for controlled cell death as well as cell survival via a regulatory role in the processes of apoptosis and cell proliferation during reptilian tail regeneration. It is possible that affected proliferation rate along with unregulated apoptosis might be the result of FGF2 signal inhibition ultimately leading to a delay in regenerative outgrowth as observed in the morphometry studies (chapter 1). FGF2 is also significant for the supporting angiogenic event of regeneration, essentially making possible the restoration of a good quality regenerate. Further, the study establishes inter-dependence between FGF2 and COX-2, two of the important players of epimorphic events, as essential during *H. flaviviridis* tail regeneration.

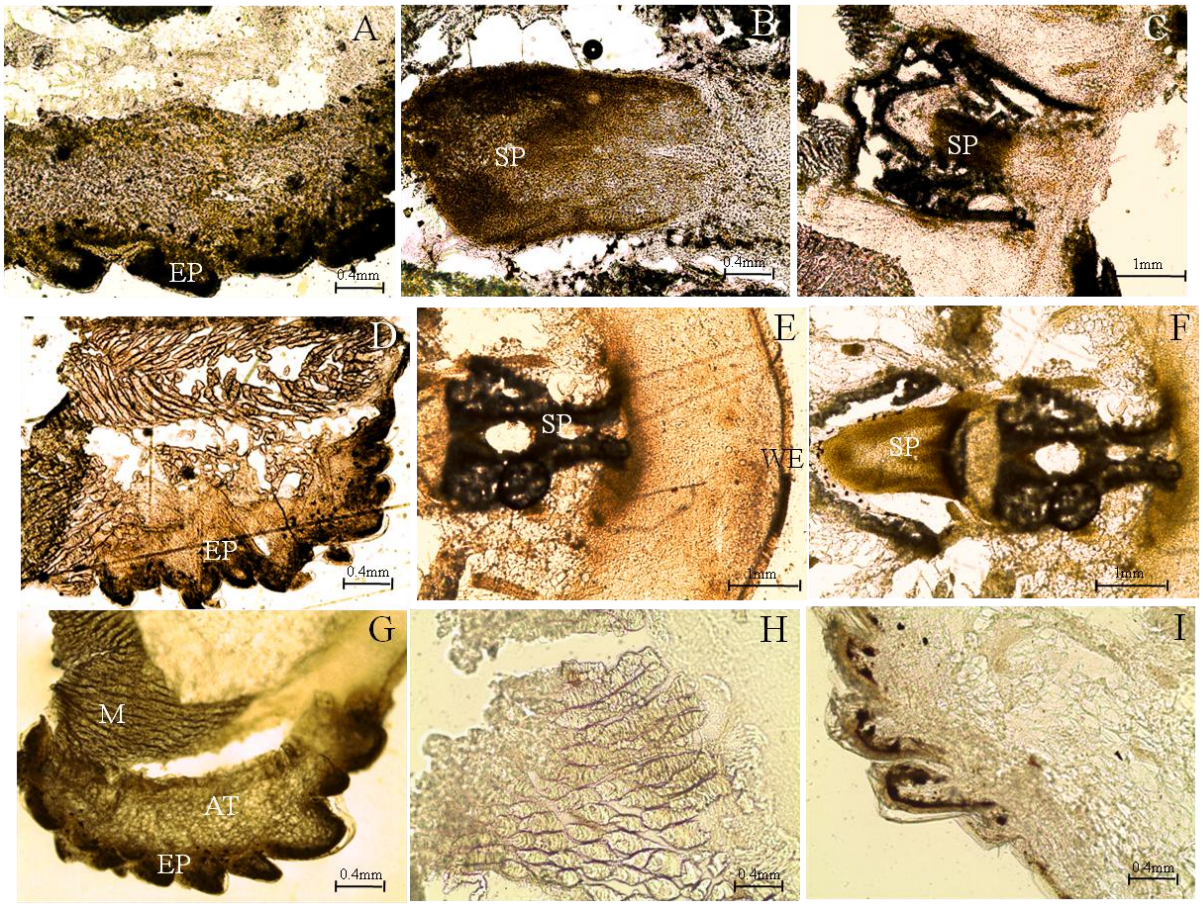
**Figure 3.1. TUNEL staining for apoptotic analysis in tail regenerates of control *H. flaviviridis***




**(A)** Apoptotic activity in the muscle bundles and the keratinized epithelium of the intact tail segment 48hpa **(B)** Wound epithelium (WE) stage with apoptosis mainly near the epithelium and nervous tissue with sparse staining in the cell accumulation prior to formation of blastema (BL) **(C)** Apoptosis in the intact tail segment at the WE stage **(D)** Localized areas of apoptosis during the BL stage **(E)** Increased apoptosis in the WE and developing muscle bundles during differentiation (DF) **(F)** Slight apoptotic activity in the ependyma at DF stage **(G)** Apoptosis still visible in the keratinized epithelium at later growth stages **(H-J)** Negative control

C- cartilage, E- ependyma, EP-epithelium, M- muscle, SP-spinal cord, WE- wound epithelium

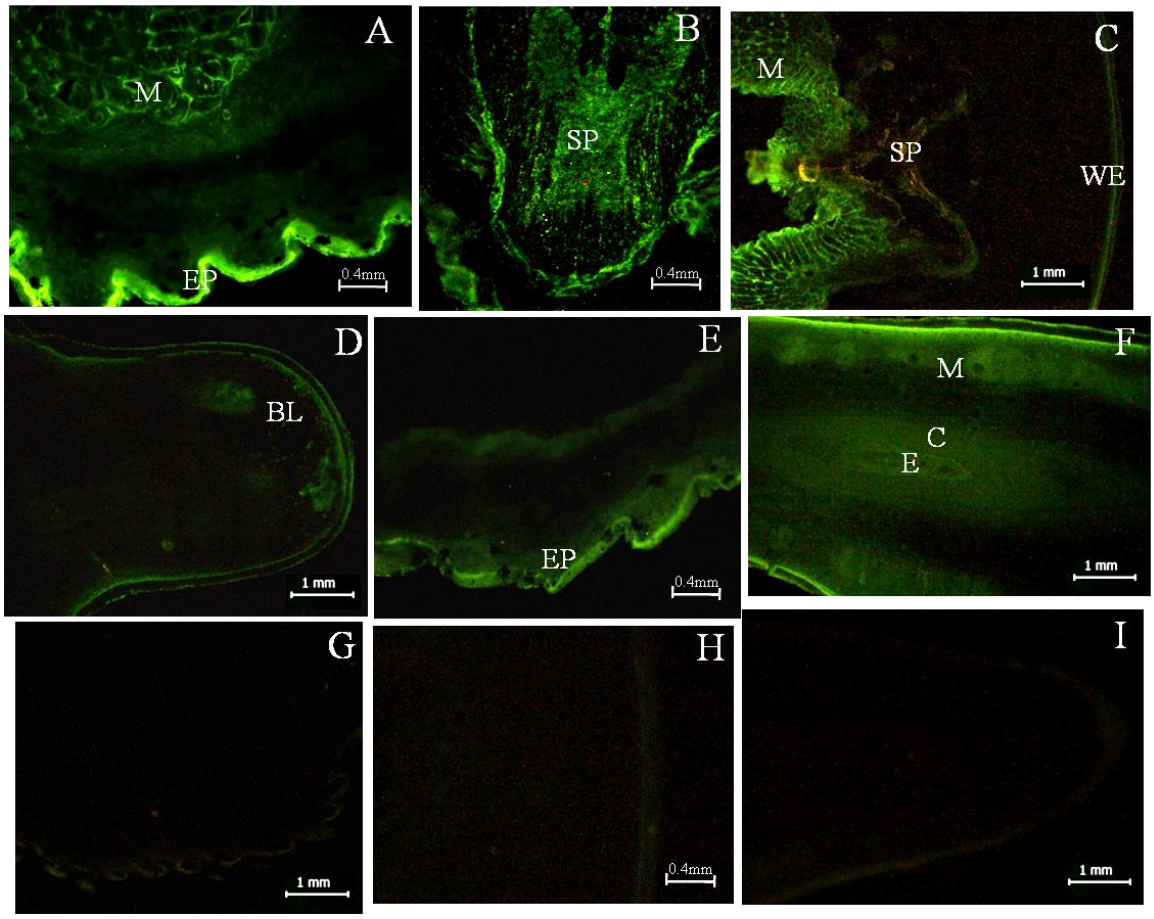
**Figure 3.2. TUNEL staining for apoptotic analysis in tail regenerates of SU5402 treated *H. flaviviridis***




**(A)** Apoptotic activity in the muscle bundles and the connective tissue of the intact tail segment 48hpa **(B)** Intense apoptosis staining in the spinal cord 48 hpa**(C)** Apoptosis after 6dpa **(D)** Apoptotic staining in the tail 9dpa **(E)** Increased apoptosis in the WE, blastemal mesenchyme beneath it and spinal cord 11 dpa **(F)** Intense apoptosis in the nervous tissue **(G)** Apoptosis in intact tail region 11dpa **(H-I)** Negative control

AT- adipose tissue, EP-epithelium, M- muscle, SP-spinal cord, WE- wound epithelium

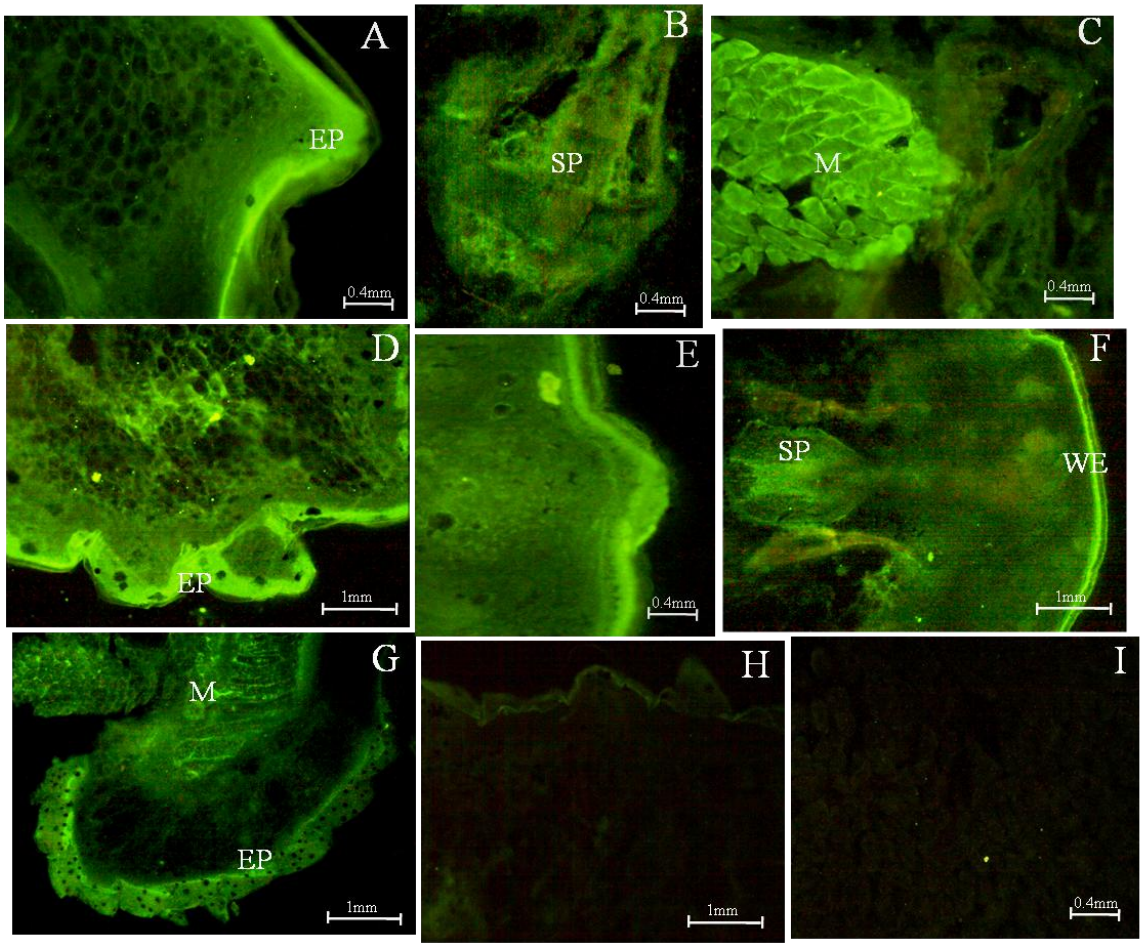
**Figure 3.3. Immunofluorescent localization of Caspase-3 during tail regeneration of control *H. flaviviridis***




**(A)** Caspase-3 activity in the epithelium, muscle bundles and surrounding connective tissue 48hpa **(B)** Localization in the spinal cord 48hpa**(C)** Caspase-3 activity in the regenerate at the wound epithelium stage and **(D)** at blastema stage **(E)** Activity in the intact tail segment region during later growth stage **(F)** Caspase-3 localized in and around the regenerating ependyma, in regenerating muscle and epithelium at differentiation stage **(G-I)** Negative control

BL- blastema, C- cartilage, E- ependyma, EP-epithelium, M- muscle, SP-spinal cord, WE-wound epithelium

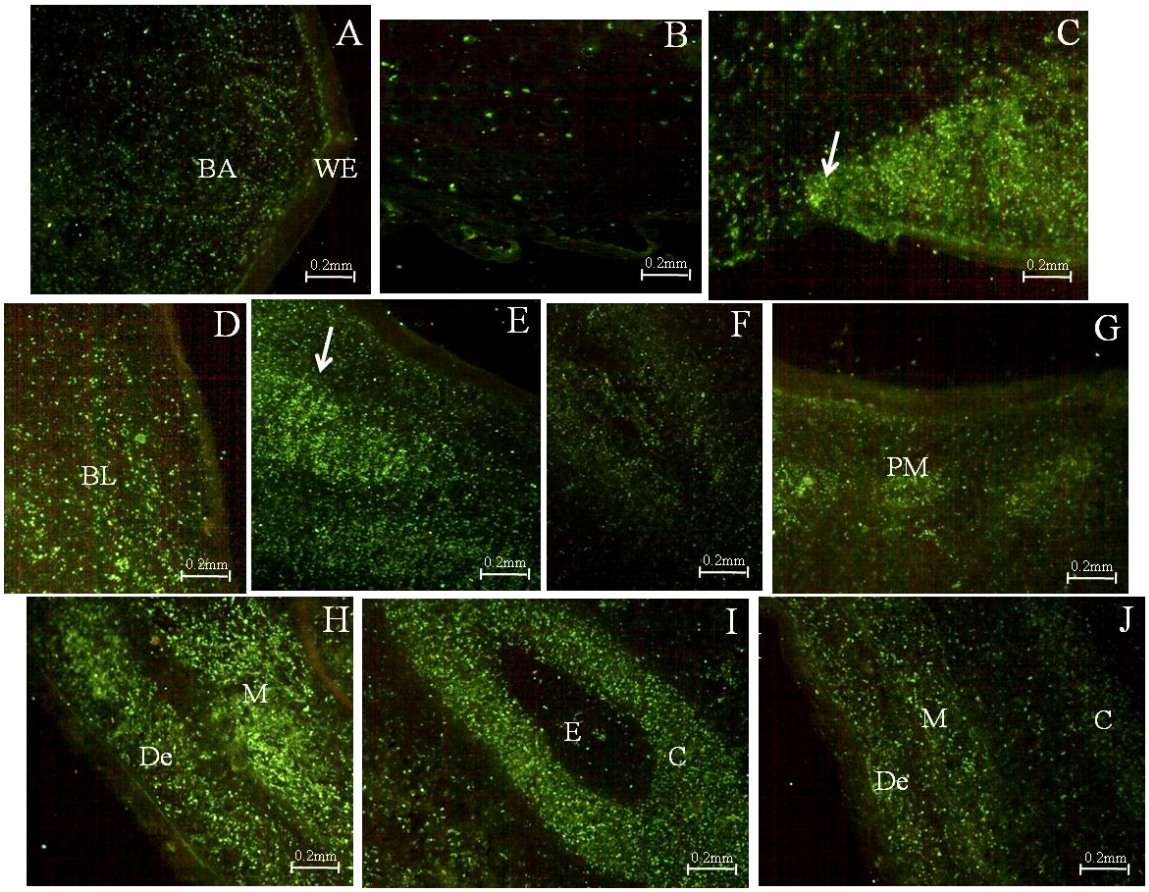
**Figure 3.4. Immunofluorescent localization of Caspase-3 during tail regeneration of *H. flaviviridis* treated with SU5402.**




**(A)** Intense Caspase-3 activity in the epithelium and connective tissue beneath it 48hpa **(B)** Localization in the spinal cord and **(C)** in muscle bundles of intact tail region 48hpa **(D, E)** Intense activity even after 9dpa **(F)** Increased Caspase-3 activity as compared to control even during later growth stage 11dpa. **(G)** Activity in the intact tail region at 11 dpa **(H, I)** Negative control

EP-epithelium, M- muscle, SP-spinal cord, WE- wound epithelium

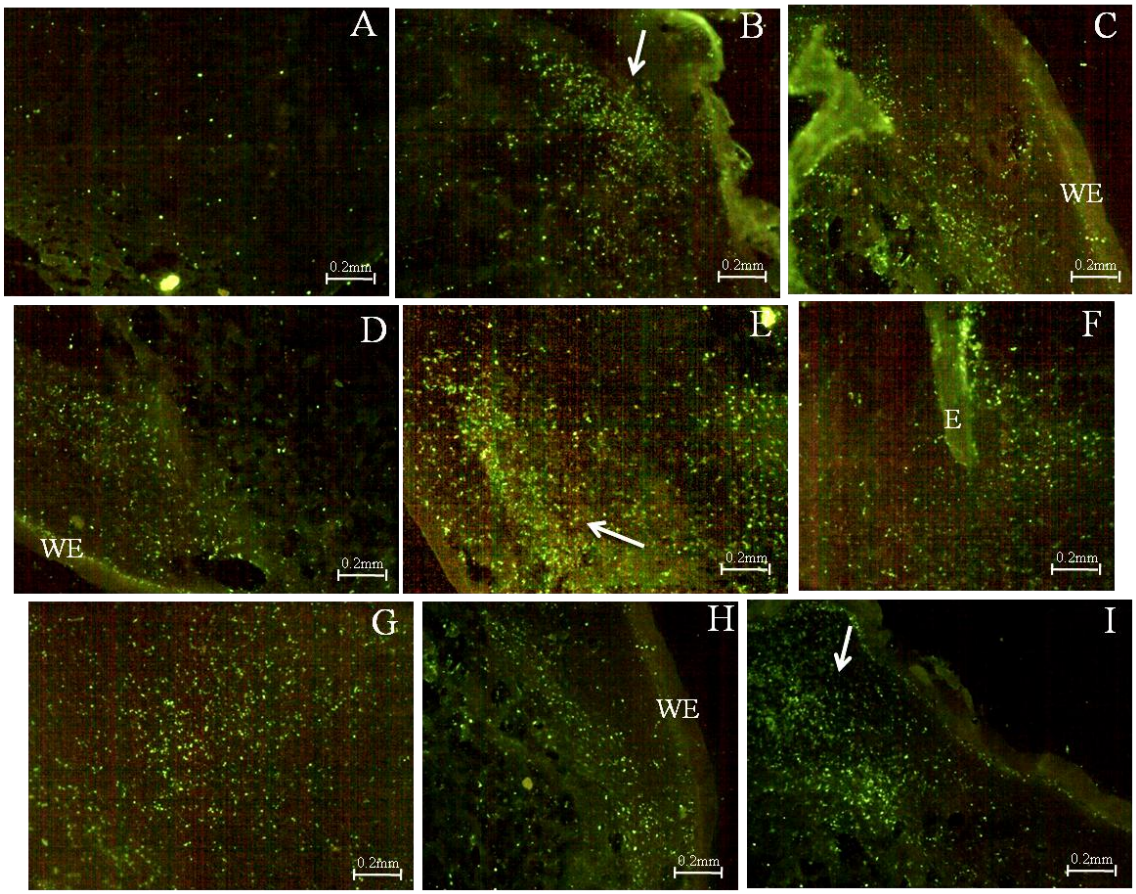
**Figure 3.5. BrdU immunolocalization in control *H. flaviviridis* during different regenerative stages.**




**(A-C)** BrdU-labelled cells at the wound epithelium (WE) stage showing labelling around the WE, cell accumulation prior to blastema and intense proliferative activity at the junction of stump and regenerate (arrow). Low number of proliferating cells in the intact tail region **(B)** can be seen **(D-G)** Labelled cells at the blastema (BL) stage, positive labelling in the blastema, at the junction of stump and regenerate (arrow), in regenerating ependyma and procartilage, and in promuscle bundles respectively **(H-J)** Proliferative activity during differentiation stage with labelled cells in regions of regenerating muscle, dermis, cartilage and connective tissue lying between muscles and cartilage

BA- cell accumulation prior to blastema formation, BL- blastema, C- cartilage, De- dermis, E- ependyma, M- muscle, PM- promuscle, WE- wound epithelium

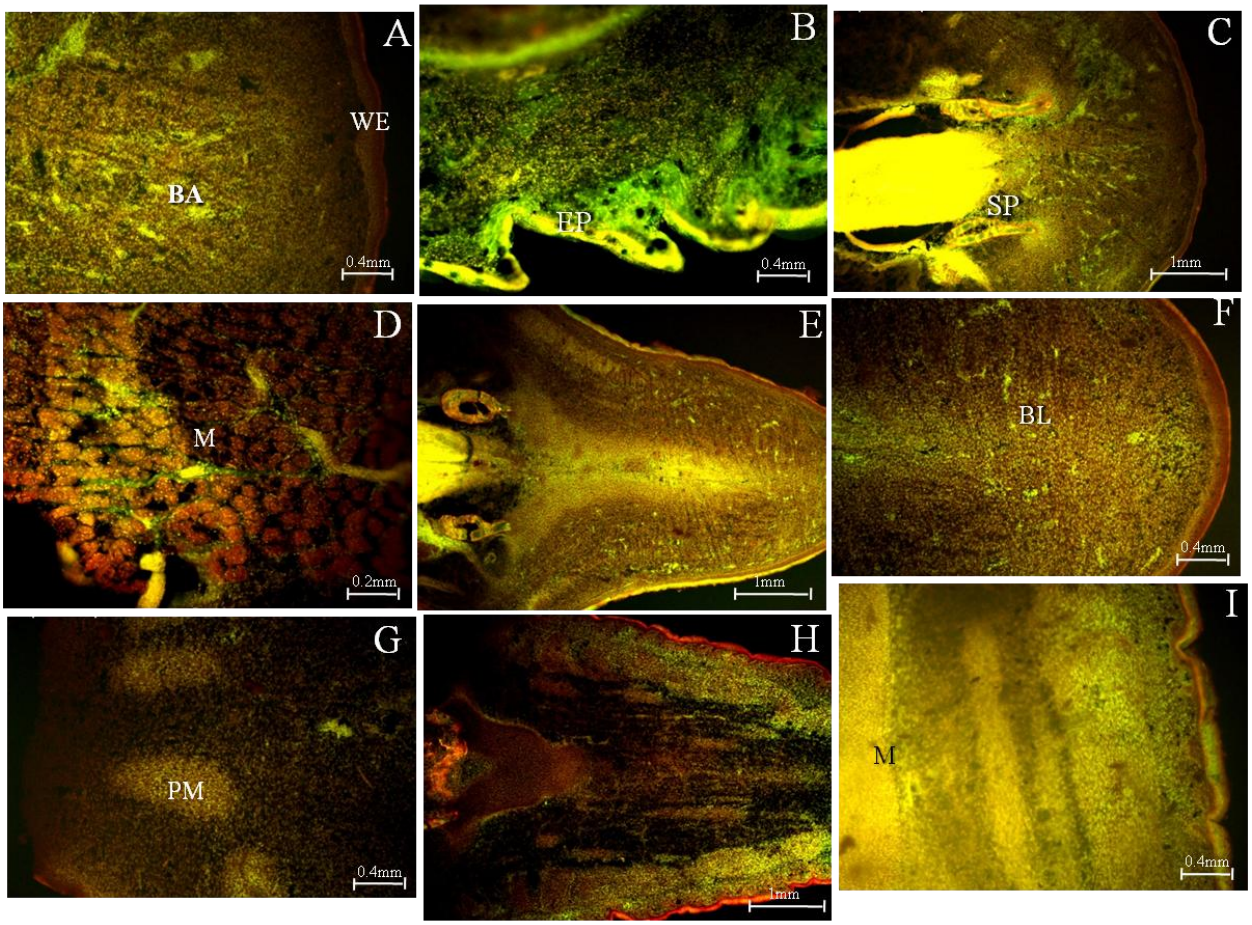
**Figure 3.6. BrdU immunolocalization in SU5402 treated *H. flaviviridis* during different regenerative stages.**




**(A-B)** BrdU-labelled cells at 6dpa, arrow indicative of region at the junction of regenerate and stump **(C-E)** Labelled cells 9dpa beneath the wound epithelium, blastemal accumulating cells and junction of regenerate and stump (arrow) **(F)** labelled cells around ependyma 11dpa **(G-I)** Proliferative activity at 11dpa showing maximum proliferation at the junction of regenerate and stump (arrow) and better proliferation in the blastema beneath the wound epithelium

E- ependyma, WE- wound epithelium

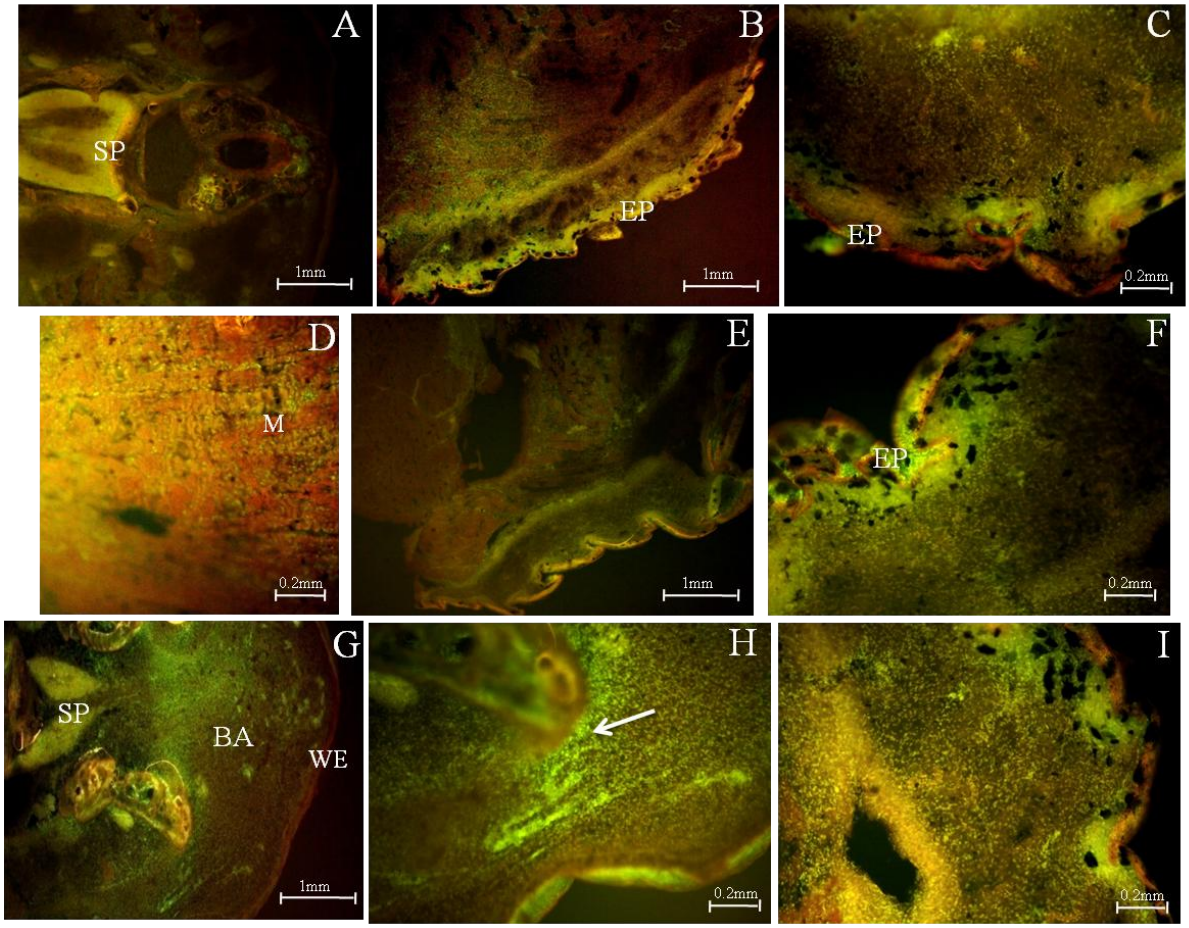
**Figure 3.7. Histofluorescent localization of nucleic acids by acridine orange staining in tail regenerates of control *H. flaviviridis*.**




**(A-C)** AO staining of nucleic acids at the wound epithelium stage in and around the WE, region of intact tail and the regenerate as a whole respectively **(D)** DNA labelling of cells among the musle bundles of the intact tail region **(E-G)** DNA and RNA fluorescence at the blastema (BL) stage in the regenerate as a whole, BL region and region in and around regenerating promuscle respectively **(H, I)** DNA and RNA staining at differentiation stage with RNA staining seen near the epithelium and intense yellow fluorescence in the regenerating tissues beneath

BA- cell accumulation prior to blastema formation, BL- blastema, M- muscle, PM- promuscle, SP- spinal cord, WE- wound epithelium

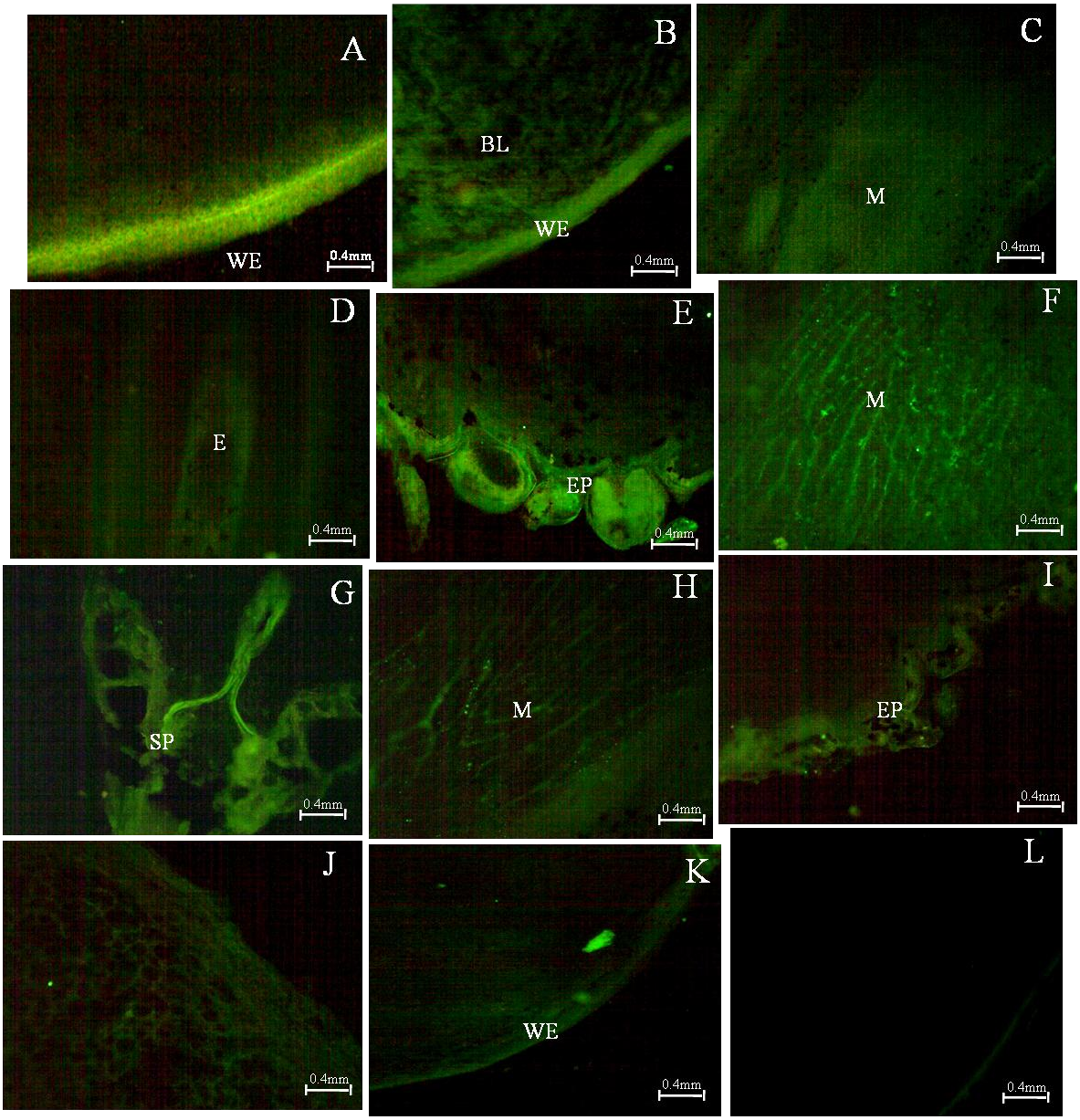
**Figure 3.8. Histofluorescent localization of nucleic acids by acridine orange staining in tail regenerates of SU5402 treated *H. flaviviridis*.**




**(A-C)** AO staining of nucleic acida 6pa **(D)** DNA labelling of cells among the musle bundles of the intact tail region **(E-F)** DNA and RNA fluorescence 9dpa **(G-I)** DNA and RNA staining at12dpa; arrow indicates junction of stump and regenerate

BA- cell accumulation prior to blastema, EP- epithelim, M- muscle, SP- spinal cord, WE- wound epithelium

**Figure 3.9. VEGF localization in tail regenerates of control and SU5402 treated *H. flaviviridis* during different stages.**

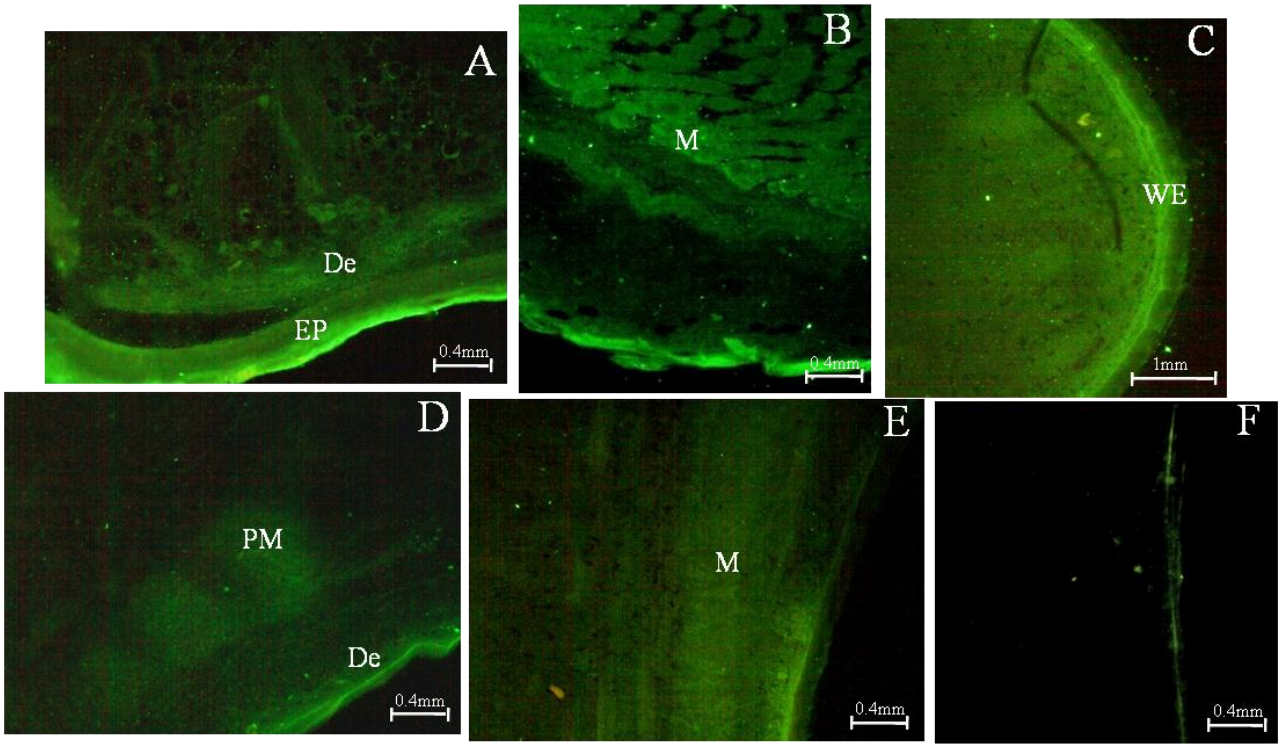



**(A- F)** VEGF in control animals **(A)** VEGF localization in the Wound epithelium **(B)** Localization in the blastemal mesenchyme and wound epithelium at blastema stage **(C, D)** Localization in the muscle bundles and region of regenerating ependyma at differentiation stage **(E, F)** VEGF activity in epithelium and muscles of intact tail region

**(G-K)** VEGF in treated animals **(G)** Spinal cord VEGF localization in treated animal 9dpa **(H, I)** Activity in muscles and epithelium of intact tail region **(J, K)** Localization in the blastemal mesenchyma and WE 12 dpa **(L)** Negative control

E- ependyma, EP- epithelium, BL- blastema, M- muscle, SP- spinal cord, WE- wound epithelium

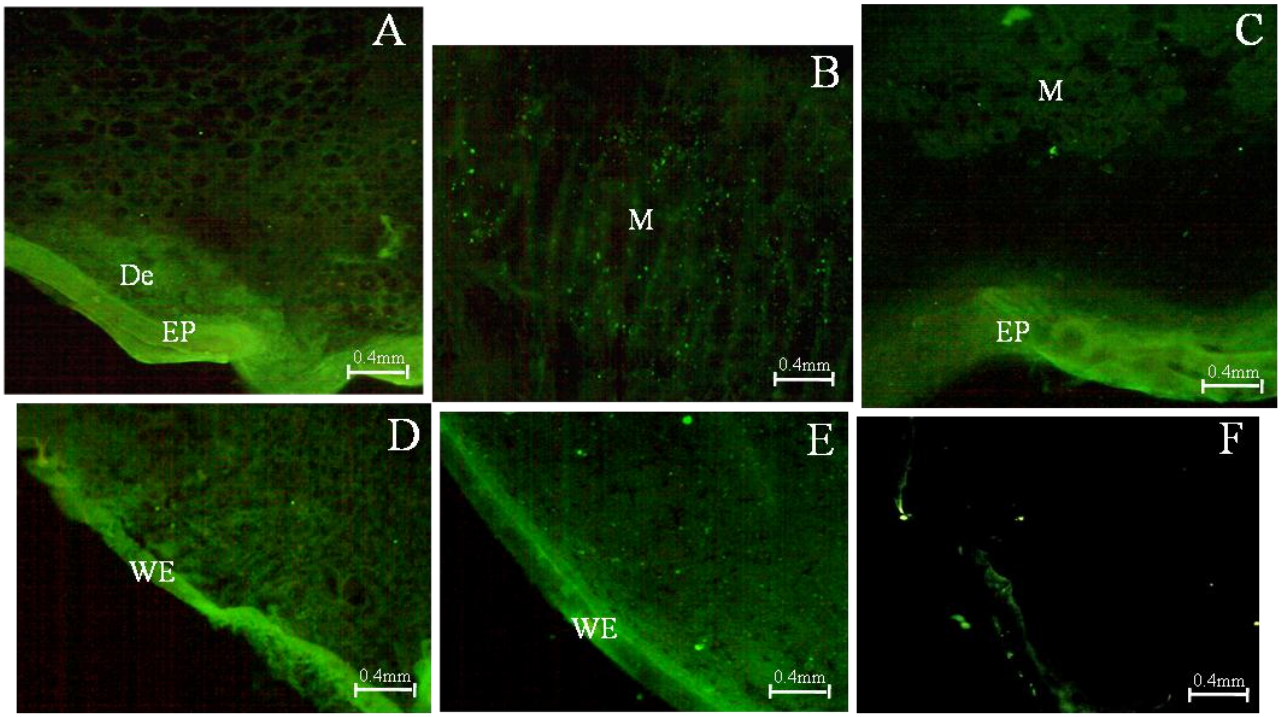
**Figure 3.10. COX-2 localization in tail regenerates of control *H. flaviviridis* during different stages.**




**(A-B)** COX-2 activity in the epithelium and dermis and connective tissue beneath it, muscle bundles of intact tail region 48hpa **(C)** wound epithelium with blastemal cell accumulation labelled at the wound epithelium stage **(D**) COX-2 in the promuscle and dermis at the blastema stae **(E)** Localization in the regenerating muscles at differentiation stage **(F)** Negative control

EP- epithelium, De- dermis, , M- muscle, PM- promuscle, WE- wound epithelium

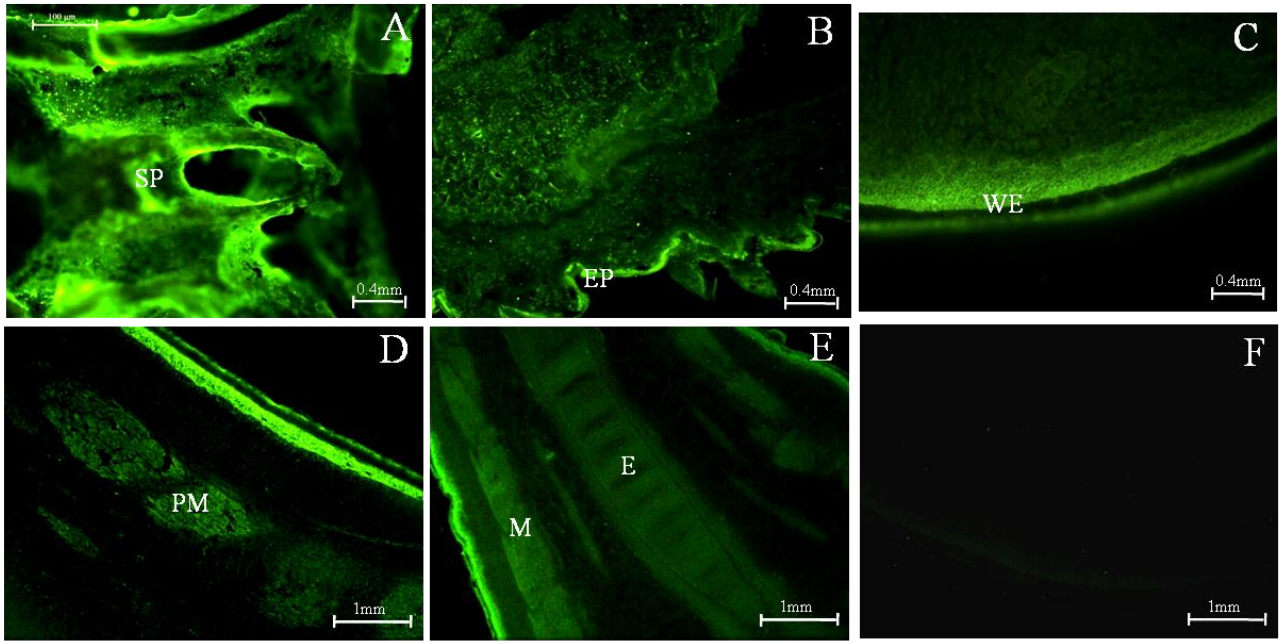
**Figure 3.11. COX-2 localization in tail regenerates of SU5402 treated *H. flaviviridis* during different stages.**




**(A-B)** COX-2 activity in the epithelium and dermis and connective tissue beneath it, muscle bundles of intact tail region 48hpa **(C)** Epithelium and muscle bundles in the intact tail region 6dpa **(D)** COX-2 in the mesenchyme and WE 9dpa and **(E)** at 11 dpa **(F)** Negative control

EP- epithelium, De- dermis, M- muscle, WE- wound epithelium

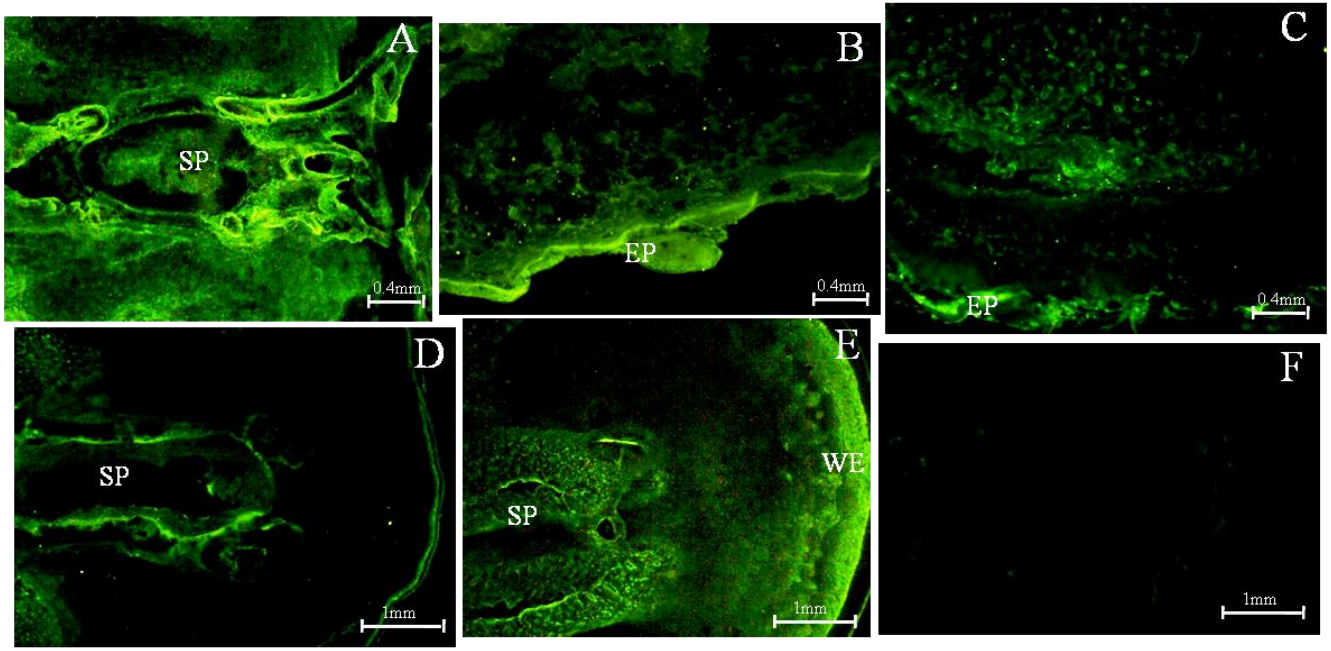
**Figure 3.12. FGF2 localization in tail regenerates of control *H. flaviviridis* during different stages.**




**(A-B)** FGF-2 localization in spinal cord region and region of intact tail 48hpa **(C)** in the wound epithelium stage **(D**) Localization in the epidermis and promuscle at blastema stage **(E)** FGF2 in the differentiating tissues at the differentiation stage **(F)** Negative control

EP- epithelium, E- ependyma, M- muscle, PM- promuscle, SP- spinal cord, WE- wound epithelium

**Figure 3.13. FGF2 localization in tail regenerates of etoricoxib treated *H. flaviviridis* during different stages.**




**(A-B)** FGF-2 localization in spinal cord region and region of intact tail 48hpa **(C)** at 6dpa **(D**) FGF2 localization in the regenerate at 9dpa **(E)** FGF2 localization after 12 dpa **(F)** Negative control

EP- epithelium, SP- spinal cord, WE- wound epithelium

# GENERAL CONSIDERATIONS

All organisms mount a biological response to damage, but they vary widely in their ability to recover. Humans constantly undergo homeostatic renewal of components of blood, skeletal muscle and epithelia and can regenerate an injured liver and repair limited insults to bone, muscle, digit tips and cornea, but they do not regenerate the heart, spinal cord, retina or limbs (Stoick-Cooper *et al*., 2007a). Thus humans and other mammals are somewhat disadvantaged when compared with organisms like amphibians, lizards and teleost fish, which have a remarkable capacity to regenerate damaged organs (Brockes and Kumar, 2002; Akimenko *et al*., 2003; Alibardi, 2010). Amphibian limbs and lizard tail provide dramatic examples of organ regeneration whereby intricate structures consisting of multiple cell types patterned into complex tissues are faithfully restored after amputation. Such restoration happens through a process called 'epimorphic regeneration' sometimes called 'true' regeneration which occurs in phases: (1) wound healing and formation of the wound epidermis; (2) formation of a regeneration blastema, a population of mesenchymal progenitor cells that is necessary for proliferation and patterning of the regenerating part and (3) regenerative outgrowth and pattern reformation (Poss *et al*., 2003). The mechanisms that enable lower vertebrates to re-establish such structures and the reasons why mammals are not able to do so are incompletely understood. Understanding the cellular and molecular mechanisms by which lower vertebrate model systems are able to faithfully regenerate complex organs holds the potential to revolutionize clinical medicine, with practical applications ranging from organ disease and wound treatment to possible alternatives to prosthetics for amputees in humans (Stoick-Cooper *et al*., 2007a).

As a result of wide-spread research being carried out in animal models which have the natural ability to regenerate, the molecules and cellular processes that play important roles in events like wound healing, inflammation, matrix reorganization, apoptosis, proliferation, differentiation and tissue patterning are now much better recognized. All these events are important processes of regeneration. Majority of such studies have been done to understand mechanisms of epimorphosis in amphibians which show an extensive power of regeneration and the same is now vividly understood (Mullen *et al*., 1996; Stoick-Cooper *et al*., 2007). However, tail regeneration following autotomy is well known in several lizard species and the process of regeneration is comparable between the lizards and amphibians (Iten and Bryant, 1976). ). 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. Understanding the mechanisms underlying this reptilian epimorphosis is important, as results can be better extrapolated to the

mammalian system since reptiles are evolutionarily closer to mammals. Further, if molecules known to be involved in amphibian regeneration play similar roles during reptilian regeneration, then regenerative mechanisms can be believed to be evolutionarily conserved across these vertebrate classes.

The precise coordination of several events involved in the complex process of regeneration requires cross talk and signalling between many factors and differential regulation of several genes. Neurotrophic factors derived from the nerve tissue are one such regulatory factors of regeneration. Most cells in the regenerating blastema- forming muscles and the regenerating epidermis are contacted by nerve terminals (Alibardi and Miolo, 1990). The trophic stimulation from spinal cord and regenerating ependymal tube have been deemed necessary to stimulate and sustain tail regeneration indicative of the importance of neurotrophic factors derived from the nerve tissue in the process (Simpson, 1964; Whimster, 1978; Alibardi *et al*., 1988). Studies on amphibian limb regeneration led to believe that FGF2 or other members of the FGF family is the neurotrophic factor required for regeneration, or is a mimic of the endogenous neurotrophic factor operating in the limb (Mullen *et al*., 1996). In fact, the FGF2 mediated rescue of denervated regenerates is the first demonstration of regeneration rescue by any means other than by nerves themselves (Singer, 1978).

FGFs are small peptide growth factors with multiple biological functions which play significant roles in patterning, growth and differentiation (Szebenyi and Fallon, 1999). A member of this family FGF2, is known to play key roles in development, tissue remodelling and disease states in almost every organ system. Involvement of FGF2 in several developmental processes such as limb development, angiogenesis, wound healing, and repair is well established (Obara *et al*., 2003; Yokoyama, 2008). Besides its many roles in several physiological and developmental processes, FGF2 is also one of the key players of epimorphic regeneration. It is known to positively influence regenerative outgrowth in several animal models such as amphibians and fish (Mullen *et al*., 1996; Hata *et al*., 1998).

FGF2 belongs to a family of at least 25 growth factors and oncogenes. 21 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 18-kDa FGF2 is the predominant isoform released by cells. 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- and cell-type-dependent (Chandler *et al.*, 1999 and Ornitz and Itoh, 2001). The biological activity of the 18-kDa FGF2 requires the presence of both heparan sulfate proteoglycans (HSPGs) and FGF tyrosine kinase receptors (FGFRs) to transduce signals for

cell proliferation (Ornitz *et al*., 1992; Guillonneau *et al*., 1996; Ornitz and Itoh, 2001). FGFRs are encoded by four distinct genes (*FGFR1–4*), and the various associations between these growth factors and their receptors regulate the specificity of FGF-induced downstream signalling and biological activities (Eshwarakumara *et al*., 2005). 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). FGF2 activates a number of intracellular signalling pathways and this occurs via binding of FGF2 to its receptors which is enhanced by cell surface HSPG leading to activation of autophosphorylation of the FGFR on several tyrosine residues. Some of the phosphotyrosine residues are binding sites for src homology 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).

Since FGF2 is implicated in a variety of growth disorders and cancers, it seems reasonable that blocking the FGF2 signal activity via inhibition of the tyrosine kinase 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. SU5402 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. The various FGFR1 isoforms have different affinities for FGFs, however, the only FGFs 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; Izikki *et al*., 2009; Lamont *et al*., 2011).

Increasing evidences being available from research on appendage regeneration in urodeles that FGF2 is one of the prime modulators of epimorphic regeneration, its influence therefore, on reptilian epimorphosis could be a certain possibility. A preliminary work done in our lab proved that FGF2 indeed influences regeneration in the reptilian model too. It was apparent from the morphometric analysis that extraneous administration of FGF2 significantly influences the wound epitheliam (WE) and blastemal (BL) stages of tail regeneration in *Hemidactylus flaviviridis* (Yadav, 2005). In order to consolidate the above findings and to further elucidate the mechanisms through which FGF2 signalling regulate the cellular events of epimorphosis in an amniote model the current study was envisaged. **The overall aim of the present study therefore, was to understand the involvement of FGF2 signalling in**

**achieving several quintessential milestones of epimorphic regeneration using northern house gecko *H. flaviviridis* as animal model.**

First, to signify the role of FGF2 during tail regeneration of *H. flaviviridis*, its immunohistochemical localization was done in the tail regenerate at key events of regeneration *viz*. wound epithelium, blastema and differentiation stages in the normal regenerating tail to learn about the tissues dependent upon this factor for their regeneration. Its levels were also quantified at these regenerative stages as well as in resting state by ELISA to get an idea of its changing levels and its requirement at different stages of regeneration. Furthermore, in order to elucidate its role in regulating progress of reptilian epimorphosis, the FGF2-FGFR1 signalling pathway was targeted using specific tyrosine kinase inhibitor SU5402 and the effects on successive stages of regeneration were observed. Also, the structural alterations occurring in the tail regenerate due to the administration of FGFR1 inhibitor were also explored through a histological study (Chapter 1).

The wound epithelium is one of the target tissues labelled intensely for this protein throughout the entire study. Blastemal cells were also labelled, though not intensely. It appears as if the action of FGF2 on wound epithelium is necessary to trigger the trophic stimulation that eventually allows blastemal cell accumulation and proliferation. Blood vessels were also labelled positively. FGF2 has many biological activities that stimulate the proliferation of fibroblast and capillary endothelial cells, thus promoting angiogenesis and wound repair (Abraham *et al*., 1986; Montesano *et al*., 1986; McGee *et al*., 1988; Gospodarowicz, 1990). FGFs have been reported to be distributed in the wound epidermis, and in particular in the apical cap of the regenerating limb of the newt, and it may stimulate the blastemal cells to induce cell replication factors (Dungan *et al*., 2002; Giampoli *et al*., 2003). An increased production of FGFs and their receptors in the wound epidermis has also been reported for mammalian skin and hairs (DuCros *et al*., 1993; Takenaka *et al*., 2002). FGF2 is present in both apical epithelial cap (AEC) and in nerves during amphibian regeneration and its levels decrease in response to denervation suggesting that there is a link between innervations and the formation of an AEC that is able to sustain distal outgrowth (Mullen *et al*., 1996). FGF2 also has a critical role for blastema proliferation and maintenance. The FGF2 receptor FGFR1 is distributed throughout blastemal mesenchyme during newt limb regeneration, suggesting that FGF2 could promote mitotic activity of blastemal cells (Poulin *et al*., 1993). It also promotes blastemal growth during zebrafish fin regeneration (Hata *et al*., 1998)

Differentiating tissues in the regenerating *H. flaviviridis* tail viz. the regenerating muscles, ependyma, nerves and cartilage also label positively for FGF2 indicating that formation and proliferation of these tissues is dependent upon this factor. Weak labelling of connective and

adipose tissues indicates a lesser role of this protein in their differentiation. FGFs have been reported to be distributed in the differentiating and growing muscle bundles of the regenerating lizard tail (Bellairs and Bryant, 1985; Alibardi, 1995). FGF2 and FGF1 are also present in developing and regenerating muscles of mammals, where these growth factors may have autocrine stimulation for their differentiation and growth (Joseph-Silver stein *et al*., 1989; Anderson *et al*., 1991). FGF2 expression is induced in the regenerating spinal cord in amphibians only after tail amputation, and is expressed in the undifferentiated cells lining the ependymal canal from which new cord will form (Zhang *et al*., 2000). FGF2 is also involved in mammalian nerve regeneration and it is up-regulated after peripheral nerve crush (Grothe and Nikkah, 2001). FGF2 is also well known as a potent regulator of functions of bone and cartilage cells. It is produced by cells of osteoblastic lineage, accumulated in bone matrix and acts as an autocrine/paracrine factor for bone cells (Canalis *et al*., 1988; Rodan *et al*., 1989, Hurley *et al*., 1994). Proliferation and differentiation of osteoblasts is also stimulated by FGF2 (Globus *et al*., 1988). Overall FGF2 is known to be distributed in the regenerating spinal cord, subset of blastemal cells, basal epidermal layer, differentiating muscles and chondroblasts (Ferretti *et al*., 2001) during amphibian limb regeneration and a similar pattern of distribution is also observed for the reptile under study. Conclusively, the mechanism by which FGF2 influences epimorphic regeneration can be said to be evolutionarily conserved among two vertebrate classes-amphibians and reptiles.

FGF2 protein levels were also measured at different stages of regeneration. High levels of this protein were recorded from the ELISA quantification after autotomy with the highest amount at the blastemal stage. FGF2 quantified during wound healing stage amounted to 80.34±0.688ng/g tissue, 96.98±2.06ng/g tissue during blastema and 75.2±1.802ng/g tissue during late regeneration. The resting level of FGF2 was approximated to 61.39±2.41ng/g tissue (Values are expressed as Mean ± S.E.). Normally FGF2 does not have a signal sequence for cell secretion through Golgi apparatus (Saksela and Rifkin., 1990; Friesel and Maciag, 1995; Dahl *et al*., 2000) and it is probably released extracellularly only after cell damage. According to this hypothesis, it is speculated that FGF2 plays a negligible role in normal stage, but with increasing damage more FGF2 is released and stimulates neurogenesis (Yoshimura *et al*., 2001). The injury to blood vessels and nerves, which occurs as a result of amputation, is thought to be a trigger for the release of FGF2. Once this preformed FGF2 is released, it further activates the synthesis and release of more FGF2 in an autocrine manner (Zhang *et al*., 2000; Yoshimura *et al*., 2001).

A subsequent decrease in FGF2 was observed with progressive regeneration and a low level was recorded in the regenerate at mid differentiation stage as compared to initial stages indicating towards the pattern of temporal requirement of this protein during regeneration. It has been reported that FGF2 inhibits skeletal muscle differentiation in chick (Kruzhkova and

Burgess, 2000). Recently it has been shown that HSPG glypican-1 acts as a positive regulator of muscle differentiation by sequestering FGF2 in lipid rafts and preventing its binding and dependent signalling (Gutierrez and Brandan, 2010). Moreover, analysis of MM14 mouse myoblasts demonstrates that terminal differentiation is repressed by pure preparation of FGF2 (Clegg *et al.*, 1987) and FGF repression occurs only during the G1 phase of the cell cycle by a mechanism that appears to be independent of ongoing cell proliferation. Further, in denervated late limbs of larval *X. laevis*, FGF2 expression at the level of amputation surface of late limbs is related to wound healing, considering that FGF2 plays a role in angiogenesis and tissue repair, but is not sufficient to promote blastema formation in the absence of FGF2 released from nerves (Cannata *et al*., 2001). Nevertheless, whether FGF2 has an inhibitory action during differentiation or the differentiated state cells are not capable of secreting sufficient amount of FGF2, both the facts indicate a lesser role of FGF2 during late differentiation and patterning.

Evidently, the current study establishes FGF2, the crucial neurotrophic factor of amphibian regeneration as a candidate neurotrophic factor of tail regeneration in a reptilian model *H. flaviviridis* as well. Hence, FGF2 inhibition studies to further analyze the mechanisms by which FGF2 regulates reptilian regeneration were explored. FGF2 signalling was inhibited by using FGFR1 inhibitor SU5402 and effects on progression of regeneration were studied. Inhibition of FGF2 signal by treatment with SU5402 hampered the wound healing process and delayed the formation of a proper wound epithelium during tail regeneration. Formation of a functional wound epithelium is essential for successful regeneration as it provides the necessary signals for the underneath tissues to dedifferentiate, proliferate and to form the blastema (Lo *et al*., 1993; Kumar *et al*., 2000). Impaired wound healing in the SU5402 treated lizards must be due to inadequate FGF2 signalling. FGF2 is a potent mitogen and is involved in epithelial cell proliferation and migration taking place during wound healing. It has also been shown that FGF2 incorporated chitosan hydrogel may be a promising wound dressing, especially in the treatment of healing impaired wounds (Obara *et al*., 2003).

Moreover, wound healing is closely complemented by cell accumulation, leading to the appearance of the blastemal cone. The proliferative blastemal cells eventually give rise to all of the cell types necessary for the complete regeneration of the lost structure (Clause and Capaldi, 2006). FGF2 is reported to be the endogenous mitogenic factor responsible for blastema formation and growth in amputated and denervated early limbs of *X. laevis* (Cannata *et al*., 2001). It regulates blastemal proliferation during fin regeneration as well (Poss *et al*., 2000; Tawk *et al*., 2002). In the current study also, SU5402 treatment, both before amputation and at WE stage, delayed this initial growth and blastema formation in *H. flaviviridis*. Evidently, the pool of accumulating blastemal cells and their proliferation leading to formation of blastemal cone was affected by impaired FGF2 signalling.

Further, once the blastema is formed, the cells get engaged in repeated cycles of cell division leading to the increase in the length of the regenerate. Injection of SU5402 before autotomy and at WE stage, curtailed the rate of growth of regenerate. A significant decrease in the length of the regenerate with 59% reduction in growth rate during 2-12 mm stage and 27% reduction during 12-24 mm stage was observed in lizards treated with SU5402 before amputation. Further, in animals treated at WE stage, a mean reduction of 46% during 2-12 mm stage and 17% during 12-24 mm stage of regeneration was observed in growth rate of regenerate. This decrease in the growth rate of regenerate was more significant from 2-12 mm. Growth rate of regenerate from 12-24mm was also decreased in the treated lizards, but it was not significant statistically. This prompted one to propose that once the regenerate accomplish a certain length and commence differentiation it no more requires FGF2 signalling for the furtherance of its growth. This notion gains credence from a stage specific treatment wherein it was observed that the growth rate of regenerate in animals that received SU5402 at blastema stage was not significantly different from that of the control animals. As discussed earlier, FGF2 is known to inhibit or at least have a lesser role during differentiation of muscle tissue (Clegg *et al.*, 1987; Kruzhkova and Burgess, 2000; Gutierrez and Brandan, 2010). Also, *in vitro*, FGF2 is known to block oligodendrocyte maturation/differentiation (Goddard *et al*., 1998). However, differentiation of certain cell types such as fibroblasts, neuroectodermal cells and melanocytes is also reportedly dependent on FGF2 (Halaban *et al*., 1992; Bhora *et al*., 1995; Gibran *et al*., 1995).

In order to complement the morphometric observations and to identify the target tissues affected by impaired FGF2 signalling, histological profile of the tail sections was also analysed in the present study. The observations revealed that several events of epimorphosis like formation of the wound epithelium, recruitment and proliferation of blastemal cells, differentiation of mesenchymal cells such as muscle bundles, ependymal growth and regeneration of the supporting cartilage surrounding the ependyma are delayed in the SU5402 treated lizards. Blood vessel formation is also evidently being downplayed in the treatment group. These results are indicative of the importance of FGF2 signalling in the timely restoration of a lost appendage with proper tissue integrity even in a reptilian model. Alibardi, (1999 and 2001) while studying limb regeneration in newts noted that the wound epidermis start as an immature keratin layer and the formation of the granulated layer occurs by the accumulation of keratohyalin-like granules. FGF2 has been reported to induce differentiation of keratinocytes (Werner *et al*., 1993). Moreover, FGF2 has been localized to the apical cap during newt limb regeneration and is reported stimulating blastemal cells to respond to cell replication factors (Giampoli *et al*., 2003).

Further, angiogenesis is tightly regulated by several extracellular signals with one of the most relevant agents known to be FGF2 (Bikfalvi *et al*., 1997). FGF2 has been detected in the basal lamina of the blood capillaries, primarily at sites of vessel branching, and in the endothelium of the capillaries of some tumours (Dimario *et al*., 1989; Cordon-Cardo *et al*., 1990) suggesting that endothelial cell derived FGF2 may mediate angiogenesis with an autocrine mode of action. There are many reports regarding the involvement of FGF2 in the regeneration of nerves during epimorphosis. It plays a role both in the early stages of regeneration, possibly in the proliferation of neural progenitors and in the maintenance of the undifferentiated state (Ferretti *et al*., 2001). It has been reported that after spinal lesion in salamander, FGF2 may be involved in functional recovery of locomotion by influencing cell proliferation and/or neuronal differentiation (Moftah *et al*., 2008). Moreover, FGF2 administration following spinal cord injury promotes hindlimb movement recovery in the adult rat (Rabchevsky *et al*., 2000). Exogenous application of FGF2 has stimulatory effects on bone formation in several *in vivo* models as pharmacological action (Aspenberg and Lohmander, 1989; Kawaguchi *et al*., 1994, Kawaguchi *et al*., 2001). FGF2 deficient mice exhibit even decreased bone mass and bone formation; although changes are rather moderate (Montero *et al*., 2000). Stimulatory effects of FGF2 on cartilage formation have also been noted in several animal models (Aspenberg and Lohmander, 1989; Kawaguchi *et al*., 1994; Nakamura *et al*., 1997).

Therefore, it is likely that FGF2 is one of the key molecules that direct the early events of the reptilian regenerative process nonetheless, its involvement, if at all, during the later stages of repatterning and differentiation is trifling. Further, it is apparent from the histological profile that the ablation of FGF2 signalling affects formation of a proper wound epithelium that in turn might affect several important signals emanating from it. Consequently, subsequent proliferation and differentiation of several tissues might get affected.

A successful wound healing response inevitably requires extensive rearrangement of cells which is possible only after regulated proteolysis of the extracellular matrix (ECM). Since FGF2 undoubtedly influences wound healing in *H. flaviviridis* tail regeneration, it might also be involved in this matrix reorganization. Proteolytic enzymes called matrix metalloproteinases (MMPs), especially the gelatin degrading proteases- MMP-2 and 9 play the most significant role in this extracellular matrix turnover event (Visse and Nagase, 2003). MMP-9 in particular is upregulated very early in the wound healing phase during amphibian regeneration and could be an important factor produced by the wound epithelium that initiates the dedifferentiation of the mesenchymal tissues (Yang and Bryant, 1994). Further, MMP degradative activity is tightly regulated by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs) (Nagase and Woessner, 1999), since excess proteolytic activity can be detrimental to regeneration. Hence, activity of these gelatinases and their endogenous

inhibitors in control and SU5402 treated animals was analyzed to elucidate the role of FGF2 in the interplay between MMP and TIMP and in the process of matrix reorganization. Gelatinases were analyzed through a zymographic study as well as their immunohistochemical localization in the tail regenerates. TIMPs were analyzed through reverse zymography. Further, biosynthesis of proteins is one of the most important biochemical processes during regeneration and it is essential to know whether FGF2 as an important regulator of reptilian epimorphosis has a role in this process of protein turnover. Hence a protein profiling for the regenerates was also done through SDS-PAGE to understand this event of regeneration (chapter 2).

Results of zymography reveal that FGF2 signalling is required for proper proteolytic remodelling of the ECM, as FGF2 inhibition significantly altered gelatinase activity, particularly MMP-2 and active MMP-9 during wound healing and blastema stages, during which gelatinase activity is required the most. Impaired gelatinase activity may be the reason for delayed cell migration, reepithelialization and WE formation, observed for the inhibitor treated lizards during morphometric studies. Delayed wound healing must also have retarded transition to the proliferative phase and hence the observed delay in attaining the blastema stage can be correlated as well. Similar reports suggesting a positive regulatory role of FGF2 on MMP activity have been published. FGF2 stimulates endothelial cell migration, pericyte attraction and matrix deposition by an increase in production of MMPs and VEGF (Presta *et al*., 2005). Stimulation of bovine nucleus pulposus cells cultured in monolayer with FGF2 augments the production of MMP-13 (potent matrix degrading enzyme) at the transcriptional and translational level in a dose-dependent manner (Li *et al*., 2008). FGF2 is also known to increase MMP-9 mRNA levels in mouse and rabbit osteoclasts. This may be important for the migration of osteoclasts through the unmineralized osteoid to reach the mineralized bone surface (Chikazu *et al*., 2000). FGF2 mediates epithelial-mesenchymal interactions of peritubular and Sertoli cells in rat testis and this is known to involve a strong induction of MMP-9 and a weak induction of MMP2 in a coculture system (Ramy *et al*., 2005).

There are reports that also suggest that ECM-growth factor interactions are bidirectional and interdependent and there exists a positive feedback loop between growth factors and MMPs (Schultz and Wysocki, 2009). For instance, MMPs influence angiogenesis (Heissig *et al*., 2003) and play an essential role in liberating growth factors and cleaving ECM proteins to reveal regions that can activate growth factor receptors (Mott and Werb, 2004). FGF2 release from the lens capsule by MMP-2 also reportedly maintains lens epithelial cell viability (Tholozan *et al*., 2007).

Low MMP expression in muscle cells underlying histolysis versus strong staining in cartilage, bone and epidermis have been observed during limb regeneration of Mexican

axolotl (Monaghan, 2009). Similar observations have been made during limb regeneration for MMP3/10b and MMP9 in the Japanese newt (Kato *et al*., 2003) and MMP9 in larval axolotls (Yang *et al*., 1999), but strong expression was observed in blastema cells for MMP9 and collagenase in the American newt (Vinarsky *et al*., 2005). Possibly the WE, bone, and inflammatory cells secrete the necessary MMPs to promote muscle histolysis and blastema formation (Monaghan, 2009). In the current study, a similar localization pattern was observed during tail regeneration of *H. flaviviridis*. Significant activity of both gelatinases could be observed in the control animals during wound healing. MMP-2 localization was prominent during blastemal and differentiation stages as in accordance with the zymogram results. Main sites of localization were the wound epithelium, especially the dermis and blastemal mesenchyme during wound healing. In addition to these, the developing ependyma and supporting cartilage tube were positively labelled during later stages. However, a slight dispersed localization was seen in the entire regenerate as well. Comparatively a lighter signal of MMP-2 activity was observed for the SU5402 treated lizards during wound healing and blastemal stages. Better labelling of MMP-9 was observed at 6 days post amputation for this group.

Further, TIMP activity is also necessary for the regenerative process. These inhibitors not only influence MMP activity but also influence cell survival and proliferation. TIMP-1and 2 have been shown to act as mitogens on a variety of human, bovine, and mouse cell types, including erythroid precursor cells, keratinocytes, fibroblasts, smooth muscle cells, endothelial cells and chondrocytes (Gasson *et al*., 1985; Bertaux et al., 1991; Nemeth and Goolsby, 1993; Hayakawa *et al*., 1994; Yamashita *et al*., 1996; Wang *et al*., 2002). These dual functions of TIMP are independent of each other, given that point mutations that abolish the proteolytic inhibitory function of human TIMP-1 do not ablate its mitogenic activity (Chesler *et al*., 1995). Human TIMP-1 has also been shown to be a cell survival factor for several cell types (Murphy *et al*., 2002; Yoshiji *et al*., 2002), as well as an enhancer or inhibitor of bone resorption depending on whether TIMP-1 concentrations are low or high, respectively (Sobue *et al*., 2001). These reports indicate towards the requirement of TIMPs to be as important as MMP activities.

Since, FGF2 influences gelatinase activity during *H. flaviviridis* tail regeneration, regulation of its inhibitor activity must also be partly under FGF2 control. Indeed, results of reverse zymography show that the TIMP levels observed for control lizards differ from those of SU5402 treated animals. Among the regenerative stages of control animals, TIMP activity was lowest at wound epithelium stage and highest at the differentiation stage (Figure 2.9). This can be correlated to the gelatinase activity observed earlier. TIMP activity for the treatment group is definitely less intense as compared to the control group, with comparatively better bands observed only during blastema stage. Altered TIMP activity may

also be one of the factors responsible for improper regenerative events observed in FGF2 signal inhibited animals. In addition to affecting MMP regulation, the potential of TIMPs to influence cell proliferation may also be impaired by SU5402. This indicates that a proper FGF2 signal is absolutely essential for optimal MMP-TIMP balance characteristic of appropriate regeneration. TIMP regulation by FGF2 is known in other systems as well. Enhanced TIMP-1 expression by FGF2 has been observed in several cell types (Overall, 1994). Similarly, FGF2 also increases TIMP-1 expression, represses TIMP-2, but does not influence TIMP-3 expression in vascular smooth muscle cells during collagen fibre modelling following vascular injury indicating towards differential regulation of TIMPs by FGF2 (Pickering *et al*., 1997). FGF2 may also be one of important regulatory factors for ECM turnover via modulation of MMP and TIMP secretion from subepithelial myofibroblasts, whereby it stimulates MMP1, MMP3 and TIMP1 secretion, but does not affect MMP2 or TIMP2 secretion (Yasui *et al*., 2004). Altogether, results of the current study show that matrix reorganization event during *H. flaviviridis* tail regeneration is evidently under the influence of FGF2 which is required for optimal proteolytic as well as TIMP activity and altered levels of both in SU5402 treated lizards provide the required evidence supporting this notion.

Further, a process like regeneration requires concerted action of several gene families such as Wnt, shh, FGF, BMP, Notch, etc. (for review see Stoick-Cooper *et al*., 2007). Regulation of differential expression of these genes as well as their gene products which are proteins forms the basis for the several events of regeneration. FGF2 being known as a major factor controlling epimorphosis at several stages, inhibition of this signalling pathway may lead to a variation in the expression of proteins. Hence an SDS-PAGE analysis of proteins of the tail regenerates was done in control and SU5402 treated lizards. Proteins of identical mobility were found in the experimental groups during all stages. Results however, evidently show that FGF2 signalling inhibition led to down regulation of several proteins in the treated animals as compared to those in the control lizards at the wound epithelium and blastema stages. Absence of certain bands with molecular mass 141.01, 41.02, 31.64, 26.05 kDa at the wound epithelium stage and bands of 127 and 19.65kDa at the blastema stage in the receptor inhibited lizards were also noticeable. During the differentiation stage, no significant differences in the relative intensity of the polypeptides between the two groups were evident.

One of the protein bands had a similar molecular weight to that of MMP-2 (66kDa) which was observed in abundance in the control animals during initial stages but found downregulated in the treated lizards. This can be correlated to the results obtained earlier in the zymography study. Besides, prominent differences in the intensity of polypeptide bands with molecular masses around 159, 102, 59 and 36 kDa between control and treatment groups were also observed during wound healing and blastema stages. Down regulation of several

other proteins in the treatment group during initial stages was also evident from the results of optical density analysis. Variations in the abundance of the polypeptides as well as absence of certain bands altogether can be attributed to the absence of proper FGF2 signalling in the SU5402 treated lizards. Impaired regeneration observed in this group can be due to the downregulation of several proteins being controlled by FGF2, essentially pointing towards its requirement for a proper regenerative response.

Combined results of the above studies conclusively indicate that FGF2 influences initial processes of wound healing as well as blastemal proliferative activities during reptilian epimorphosis. Its localization in several differentiated tissues as well as impaired differentiation of these in FGF2 signal inhibited animals point towards its role in initial differentiation as well, though its influence on later morphogenesis and patterning appears to be trivial. Hence it is reasonable to suppose that FGF2 might be involved in regulating several cellular events like apoptosis, proliferation as well as angiogenesis during *H. flaviviridis* tail regeneration contributing to the formation of a proper regenerate. This hypothesis was tested by studying these processes in control and FGF2 signal inhibited lizards. Apoptosis was evaluated through a TUNEL assay as well as localization of caspase-3 in tail regenerates. Proliferative activities were analysed through *in vivo* BrdU incorporation and its subsequent localization as well as acridine orange staining of the regenerates. Localization of VEGF in the regenerates was done to understand the role of FGF2 in regulating angiogenesis. Further the relation of FGF2 with another important regulator of regeneration, the enzyme cyclooxygenase-2 was explored, considering some theories which suggest that FGF2 works through COX-2 and prostaglandin pathway (Bikfalvi *et al*., 1997, Foegh and Ramwell, 2004) and conversely some reports suggest that cyclooxygenases regulate FGF2 mediated proliferation and angiogenesis (Antoniotti *et al*., 2003; Finetti *et al*., 2008). Hence, a localization study of COX-2 in FGF2 inhibited animals and of FGF2 in COX-2 inhibited animals was done during *H. flaviviridis* tail regeneration to elucidate the interaction between FGF2 and COX-2 during this process (chapter 3).

A certain degree of cell death by apoptosis is an important feature of any morphogenetic process, be it development or regeneration. Apoptosis is not only required to clear the damaged cells but this regulated cell death may also be essential for balancing the process of extensive cellular proliferation and useful for tissue patterning which are characteristic of any morphogenetic event. In fact, an endogenous early apoptotic event is reported to be required for regeneration despite the massive tissue proliferation involved (Tseng *et al*., 2007). Whether FGF2 is a factor that mediates control of such an event during *H. flaviviridis* tail regeneration is not known. Since FGF2 inhibition delayed the regenerative event and also affected differentiation of several tissues, its role if any, in regulating the apoptotic event during reptilian epimorphosis was evaluated. From combined results of TUNEL staining and

Caspase-3 localization in tail regenerates of control and SU5402 treated lizards; it could be observed that FGF2 signal inhibition certainly leads to unregulated apoptosis of cells during regeneration. A greater degree of apoptosis was observed in the early stages after amputation and this decreased as the regeneration progressed with an appreciable but not intense level of apoptosis again seen at the differentiation phase in the control group. Compared to control animals, SU5402 treated animals showed an elevated level of cell apoptosis during all stages of experiment, with unregulated cell death occurring even after 11-12 day post amputation. Nevertheless, sites of cell death remained the same in both the groups which included the spinal cord, muscle tissue as well as region near the epithelium. Higher levels of apoptosis at all these sites were observed for the treated group. Controlled apoptosis in the differentiating tissues was also observed during later stages for the control group. It is possible that FGF2 signal inhibition led to this increased cell death, since FGF2 is known to be a cell survival factor and greater apoptotic activity in this group led to delayed regeneration as well as affected morphogenesis causing poor histoarchitecture.

FGF2 is a known mitogen and one of the mechanisms by which it augments cell proliferation might be the inhibition of apoptosis. Reportedly, Bcl2 family proteins are targets of FGF2 signalling in mediating cell survival by regulating apoptosis (Bryckaert *et al*., 1999; Kim *et al*., 2012). The protein ccp1, a downstream target of FGF2 signalling is known to regulate cell proliferation and apoptosis in neuroblastoma cells (Pellicano *et al*., 2010). FGF2 mediated *survivin* expression inhibits apoptosis through direct interaction with caspase-3 in small cell lung cancer cells (Xiao *et al*., 2008). In contrast, FGF2 is also known to induce apoptosis in certain cells like rat myofibroblasts (Funato *et al*., 1997), neural retinal cells in developing chicks (Yokoiyama *et al*., 1997), human breast cancer cells (Wang *et al*., 1998) and in differentiating osteoblasts (Mansukhani *et al*., 2000). FGF2 is also known to differentially regulate members of the TNF superfamily of death domain receptors and their ligands (Eves *et al*., 2001). Nevertheless these findings are indicative of the role of FGF2 in regulating cell death and survival.

As seen previously, FGF2 signalling is essential for blastemal proliferation and subsequent tail outgrowth as both these processes were hampered in SU5402 treated lizards. Evidently FGF2 affects rate of cell proliferation between the two groups. This hypothesis was tested by *in vivo* BrdU incorporation and subsequent localization of BrdU labelled cells. Results showed a lower cell proliferation rate in FGF2 signal inhibited animals during all the proliferative stages of regeneration studied. Evidently, FGF2 confers a proliferative potential to the blastemal cells during reptilian regeneration. This is in accordance with regeneration studies in other animal models such as newts and zebrafish, reporting a similar mitogenic influence of FGF2 on blastemal cells and causing regulated cell proliferation. During amphibian tail regeneration, FGF2 has been shown to increase the proliferation of cells and

accelerate the regeneration process (Poulin *et al*., 1993; Hata *et al*., 1998; Ferretti *et al*., 2001). It has also been suggested that during urodele limb regeneration, FGF2 produced in the limb mesenchyme could act as an autocrine factor stimulating blastemal cell proliferation and as a paracrine factor promoting in the permissive epidermis (AEC) the synthesis of several forms of FGF that in turn promote blastema growth and pattern formation (Stoick-Cooper *et al*., 2007).

The proliferative role of FGF2 might be due to its direct effect on the synthesis of DNA, which is needed by rapidly dividing cells of the regenerate. In fact FGF2 is known to be an active participant during cell cycle (Korr *et al*., 1992; Liu *et al*., 1997) and induces a single re-entry of G0 rat astroglial cells into the mitotic cycle. Zeitler *et al*. (1997) have reported that FGF2 induces cell cycle progression from G0/G1 to S phase in endothelial cells as well. Regulation of proliferation is also essential and FGFs provide this by controlling the length of the G1 phase. Addition of FGF2 in primary cortical neuron culture derived from mouse at E14-E16 showed shortening of the G1 length and increase in proliferative divisions, indicating that FGF2 controls cell proliferation via its control of G1 length (Lukaszewicz *et al*., 2002). Besides, FGF2 is known to be mitogenic for many different cell types both *in vitro* and *in vivo* (Richards *et al*., 1992; Hoppenrejis *et al*., 1994; Martin *et al*., 1997). FGF2 has also been implicated in several cancers (Kormann *et al*., 1988; Giri *et al*., 1999). Inhibition of either FGF2 or FGFR1 reduced ERK1/2 activation, cell proliferation, and survival in uveal melanoma cells (Lefevre *et al*., 2009). Contrary to this, FGF2 inhibits proliferation of rat chondrosarcoma cells and arrests cell cycle at G1 phase (Aikawa *et al*., 2001). Thus FGF2 promotes cell proliferation in some cell types, while inhibits in others. Nuclear translocation of FGF2 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).

Since FGF2 conclusively plays a role in influencing rate of proliferation during reptilian regeneration, further, in support of this result, acridine orange staining of the nucleic acids in the regenerates was done. Qualitative differences in the levels of nucleic acids between control and treatment group is very much evident from the results obtained. Control group evidently has better DNA and RNA fluorescence in the regenerate during various proliferative stages as compared to SU5402 treated lizards which show good fluorescence only after 12-13 days post amputation. The level of nucleic acids though not indicative of proliferative activities but can be correlated to it, given that an active process like proliferation occurring during regeneration can be related to processes of DNA replication and subsequent transcription and translation of several proteins to regulate such a process and meet the demands of the rapidly dividing cells and considering the results obtained, FGF2 definitely seems to be involved in these molecular processes related to proliferation.

Another important observation from the proliferation studies was that a significant number of dividing cells are present at the junction of the stump and the regenerate during all the stages of regeneration studied. This region also showed intense nucleic acid staining with acridine orange. This region seems to provide a continuous input of cells into the regenerate and hence proliferative activities of resident stem cells in this mesenchymal zone may be significantly responsible for subsequent blastema formation and restoration of the lost tail. Nevertheless, it is important to note that proliferative activities in this region also, both BrdU labelled cells and nucleic acid localization, are significantly affected by FGF2 signal inhibition.

Since, SU5402 affected growth rate as well as quality of the regenerate; it was worth evaluating the regulation of angiogenic process by FGF2 during *H. flaviviridis* tail regeneration. This was done through localization of vascular endothelial growth factor (VEGF) in the regenerates of control and SU5402 treated lizards. VEGF is an important angiogenic regulator and its signalling stimulates the survival of endothelial cells, their proliferation and their motility, initiating the sprouting of new capillaries (Bruick and McKnight, 2001). Results of this study show that activity of VEGF is definitely downregulated by FGF2 signal inhibition. Localization of this molecule in several tissues of the regenerate was comparatively less intense in the FGF2 signal inhibited animals. VEGF inhibition is known to affect morphogenesis and tissue patterning, but does not abolish regeneration (Shibuya, 2006). Hence it is possible that the poor histoarchitectural features observed in the SU5402 treated animals is induced by VEGF downregulation due to FGF2 signal inhibition. Differences in VEGF activity in control and treated animals being very much evident, it can be concluded that FGF2 is definitely required for VEGF mediated angiogenesis during reptilian regeneration. Moreover, FGF2 being a potent angiogenic molecule, its inhibition in itself is a limiting factor for formation of a proper regenerate. Besides, there are several reports suggesting that FGF2 regulates VEGF activity and angiogenesis. It induces VEGF expression in the endothelial cells of forming capillaries through autocrine and paracrine mechanisms (Seghezzi *et al*., 1998) and causes neovascularisation indirectly by activation of VEGF/VEGFR system (Presta *et al*., 2005). Endothelial cell tube formation stimulated by VEGF in murine embryonic explants, depends upon endogenous FGF2 (Tomanek *et al*., 2001). Similarly, FGF2 antibodies have also been shown to block VEGF-induced angiogenesis *in vitro* (Mandriota and Pepper, 1997). Proliferation and differentiation of VEGFR-$2^{+}$ hemangioblast precursors from the mesoderm (Poole *et al*., 2001) is promoted by FGF2. FGF2 can also induce vascular permeability directly and indirectly by upregulating VEGF and proteases and this has a role during inflammatory phase of wound healing (Reuss *et al*., 2003).

Besides FGF2, another important inducer of angiogenesis is the cyclooxygenase enzyme COX-2. COX-2 is an inflammatory mediator that is involved in wound healing and tissue

repair besides angiogenesis (Kurie and Dubois, 2001; Murakami and Kudo, 2004; Wilgus *et al*., 2004; Shen *et al*., 2006). There are several reports showing a direct association between COX-2 and FGF2 mediated signalling and these reports suggest a two-way regulation of this interaction. FGF2 induces prostaglandin synthesis in a variety of cell types, often in context of angiogenesis or tissue healing (Kawaguchi *et al*., 1995; Kage *et al*., 1999; Hosono *et al*., 2011). FGF2 administration in retinal epithelial cells is known to cause an increase in COX-2 mRNA (Ershov and Bazan, 1999). COX-2 and hypoxia regulated proteins are modulated by FGF2 in acute renal failure (Villanueva *et al*., 2012). FGF2 accelerates astrocyte migration in a scratch model *in vitro* which is a key factor in the repair mechanisms orchestrated by FGF2 and this effect is partly mediated by an upregulation of COX-2 (Lichtenstein *et al*., 2012). Conversely, COX-2 inhibition suppresses FGF2 expression in human oesophageal adenocarcinoma (Baguma- Nibasheka *et al*., 2007). Inhibition of COX-2 by celecoxib delays tumour growth and metastasis in xenograft tumour models as well as suppresses FGF2 induced neovascularization of the rodent cornea indicating that FGF2 activity depends upon COX-2. Over expression of COX-2 which characterizes many epithelial tumours as well as their endothelial population, is accompanied by enhanced expression and production of angiogenic factors like VEGF and FGF2 (Abdelrahim and Safe, 2005; Larkins *et al*., 2006). Moreover, FGF2 and VEGF mediated angiogenesis is partly dependent on increasing expression of COX and $PGE_2$ production (Hernandez *et al*., 2001; Salcedo *et al*., 2003).

The association of COX-2 and FGF2 signalling pathways is very much evident from these reports. Considering this fact, whether a co-ordinated regulation of these factors exists during *H. flaviviridis* tail regeneration was evaluated by a two way study. Influence of FGF2 inhibition on COX-2 activity and vice versa was analyzed by a localization study of COX-2 and FGF2 in the tail regenerates respectively. Results show that SU5402 did not substantially affect COX-2 activity during regeneration when compared to control group. However, its localization in muscle region definitely seems to get affected. Conversely, COX-2 inhibition by etoricoxib was found to significantly affect FGF2 localization during regeneration. Obvious differences in staining intensity could be observed between the control and treatment groups in several areas of the regenerate. These results suggest that FGF2 may not largely influence COX-2 mediated signalling during regeneration of the studied animal model. However, COX-2 activity evidently seems to be necessary for FGF2 expression and subsequent signalling. As such there are reports that COX-2 induced $PGE_2$ is an important regulator in the processes of wound healing, proliferation, myogenesis and angiogenesis in the same animal model (Sharma and Suresh, 2008; Suresh *et al*., 2009), and its inhibition leads to improper and delayed regeneration. Evidently it also must be regulating FGF2, which itself is a prerequisite for a successful regenerate formation.

Conclusively, from the current study it is apparent that FGF2 signalling is quintessential for the early events of epimorphosis in northern house gecko *Hemidactylus flaviviridis*. This growth factor, on possible induction by the inflammatory mediator $PGE_2$, invokes widespread matrix reorganization that is essential for the recruitment of pleuripotent mesenchymal cells (blastema) at the site of amputation by a controlled regulation of TIMPs and MMPs. Further, it can be deduced that the optimal FGF2 signalling is indispensable for controlled cell death as well as cell survival since it has been observed that the impairment of FGF signalling deranged the normal pattern of apoptosis and cell proliferation during reptilian tail regeneration. Moreover, FGF2 signalling is also found essential for the supporting angiogenic event of regeneration, essentially making possible the restoration of a good quality regenerate. Further, the study establishes the cross-talk between FGF2 and COX-2, two of the important regulators of epimorphic events, as essential during *H. flaviviridis* tail regeneration. Nevertheless, many of the downstream proteins which were found altering in their expression level during the course of tail regeneration need to be characterised and identified through a comprehensive proteomic analysis to have a clear comprehension of the coordinated orchestration of molecular signalling during tail regeneration. Studies have been initiated in this direction and it is hoped that one could unwind the mystery behind the complex regulation of epimorphosis in the time to come but never to undermine the significance of current study that helped us think in this direction.

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