ROLE OF NEURAL PEPTIDES AND GROWTH FACTORS ON EPIMORPHIC REGENERATION:

INFLUENCE OF FGF-2 AND EGF ON LIZARD TAIL REGENERATION

[CONCISE SUMMARY]

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CONCISE SUMMARY

Regeneration is a fundamental attribute of all living things, whether it is simple tissue restoration or the complete replacement of lost body parts, such as limbs, tails or even heads As a biological problem, it began to be formally studied over 250 years ago in crustaceans by Rene-Antione Ferchault de Reaumur (1683 -1757), and soon after in *Hydra* by Abraham Trembly (1710-1784) In the years to come, regeneration attracted the attention of prominent biologists such as Charles Darwin (1809-1882) and Thomas Hunt Morgan (1866 –1945) A long-standing problem of biology, regeneration in metazoans still awaits a satisfactory mechanistic explanation. The Italian Scientist Lazzaro Spallanzani (1729-1799) investigated in great detail the ability of various animals to regenerate missing body parts In his "*Prodromo di un opera da imprimersi sopra la riproduzioni. animali*" published in 1768, Spallanzani reported the regenerative properties of earthworms and snails, and of the legs, tails and jaws of aquatic salamanders, as well as tails of tadpoles and legs of the young and adult frogs. Further, throughout the history of experimental biology, certain organisms have repeatedly attracted the attention of each new generation of researchers. These "fascinating" animals are unique because they point out the potential of biological processes as they apply to humans Urodele amphibians are members of this group because they alone, among the vertebrates, are able to regenerate lost body parts as adults They are a constant reminder that regeneration is an ancient and fundamental biological process, and they challenge our creative and scientific abilities to discover how to unlock the regeneration potential within us Though, the ability to regenerate is tremendous in invertebrates, among the vertebrates, the most extensive power of regeneration is shown by amphibians, wherein Urodeles can regenerate their limbs, tails, jaws and snout while Anurans can regenerate their hind limbs 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)

Based on cellular mechanisms, regeneration can be divided into two broad categories. 1) morphallaxis and 2) epimorphosis Morphallaxis refers to the type of regeneration in which lost body parts are replaced by the remodeling of the remaining tissue Nearly all organisms that regenerate do so through the utilization of stem cells or progenitor cells In many cases these stem cells are already present in the organism and need only be activated when the

need arises For example, *Hydra* utilizes stem cells found in the gastric region to regenerate itself or its lost structures (Bosch, 1998) and thus is considered immortal (Chatterjee *et al.,* 2001) This process is not dependent upon cellular proliferation In contrast to morphallaxis, epimorphosis requires active cellular proliferation prior to the replacement of the lost body part For example, planarians use pre-existing stem cells, known as neoblasts, to regenerate lost structures (Morgan, 1898; Morgan, 1901) This remarkable regeneration ability depends on the activation, proliferation and differentiation of neoblasts (New mark *et al.,* 2000) However, in vertebrates, epimorphic regeneration is the most prevalent form of regeneration and also the most widely studied In vertebrates with more extensive power of regeneration, such as salamanders, new stem cells or progenitor cells are created through a process of cellular dedifferentiation in which differentiated cells can reverse the normal developmental processes and once again become precursor cells (Thornton, 1938, Thornton, 1938; Chalkley, 1954, Bodemer *et al.,* 1959, Hay *et al.,* 1961, Lo *et al.,* 1993; Kumar *et al.,* 2000) In mammals, however, stem cells are activated during regeneration of muscle, bone, epithelia and blood and recently it has been demonstrated that certain regions of the adult mammalian central nervous system also contain stem cells (Uchida *et al.,* 2000)

The process of epimorphic regeneration has been widely studied in amphibians, where it occurs in several stages like. 1) preblastemal or wound epithelial stage, 2) blastemal stage; and 3) differentiation or morphogenetic phase Following limb amputation in amphibians, epithelial cells begin to migrate across the amputation site to form wound epithelium (WE), which is a few layers thick This WE thickens in response to continued epithelial cell migration and within days forms the mature Apical Epithelial Cap (AEC) (Christensen and Tassava, 2000) The internal stump cells underlying the WE/AEC begin to dedifferentiate in response to undefined signals found in the early limb regenerate (Chalkley, 1954; Thornton, 1957, Bodemer *et al.,* 1959; Hay *et al.,* 1961, Thornton *et al.,* 1965; Steen, 1968, Lo *et al.,* 1993, Kumar *et al.,* 2000) These dedifferentiated cells then proliferate to form a mass of progenitor and pleuripotent cells, known as regeneration blastema, which harbours the cells that will later redifferentiate to form the regenerated limb Some of the dedifferentiated cells can even transdifferentiate and contribute to cell lineages other than the lineage of origin (Steen, 1968, Namenwirth, 1974) More recent experiments have clarified the degree of cellular plasticity that can occur during *Salamander* limb regeneration (Lo *et al.,* 1993; Kumar *et. al.,* 2000). Besides, several experiments have led to the conclusion that fully differentiated newt muscle cells can transdifferentiate into chondrocytes through a dedifferentiation/redifferentiation process and is consistent with the hypothesis that some dedifferentiated cells might be multipotent (Brockes and Kumar, 2002) Thus, regeneration proceeds by local reversal of differentiation of adult tissues to provide the proliferating mesenchymal or epithelial progenitor cells of the regenerate (Brockes, 1994, Okada, 1991,

Wallace, 1981). During limb regeneration in amphibians, the blastemal progenitor cells arise by dedifferentiation of mesenchymal tissues at the amputaiton plane (Hay 1959, Steen 1968, Namenwirth, 1974, Lo *et al.,* 1993)

Dedifferentiation is also evident during the regeneration of other structures and organs For example, Salamander jaw regeneration requires the dedifferentiation of the same types of cells that dedifferentiate during limb and tail regeneration (Ghosh *et al.,* 1995; Ferretti *et al.,* 1997). During regeneration of newt retina or lens, the pigmented epithelial cells of the retina or dorsal iris, respectively, dedifferentiate to form proliferating non-pigmented precursor cells that dedifferentiate to form the lost structure (Ito *et al.,* 1999). Similarly, during the early stages of spinal cord regeneration in amphibians, the ependymal cells that line the central canal of the spinal cord transform from an epithelial to a proliferating mesenchymal cell type that will later redifferentiate not only into ependymal cells but also into interneurons that comprise the gray matter of the regenerated spinal cord (Butler *et al* , 1967; O'Hara *et al.,* 1992). Moreover, in response to cardiac injury, newt cardiomyocytes dedifferentiate to a proliferative state where they disassemble their myofibrils, reenter the cell cycle, and proceed through mitosis and cytokinesis (Oberpriller *et al.,* 1971, Oberpriller *et al* , 1974, Bader *et al.,* 1978, Badar *et al* , 1979, Oberpriller *et al.,* 1995, Neff *et al.,* 1996)

However, cellular dedifferentiation, a phenomenon central to epimorphic regeneration in newts, is not normally observed in terminally differentiated mammalian myotubes. Mouse myotubes are incapable of reentering the cell cycle, unless they have been genetically altered or treated with myoseverin, a microtubule binding purine (Endo *et al* , 1989; Iujvidin *et al.,* 1990, Schneider *et al.,* 1994, Tiainem *et al.,* 1996; Novitch *et al.,* 1996; Endo *et al.,* 1998; Rosania *et al.,* 2000) Ectopic expression of msx-1, which encodes a homeobox-containing transcriptional repressor, can induce mouse myotubes to dedifferentiate to mononucleated cells that posses the properties of stem cells (Odelberg *et al.,* 2000) Moreover, mouse myotubes when treated with newt regeneration extract, are induced to dedifferentiate (Christopher *et al* , 2001), while cultured newt myotubes can dedifferentiate when transplanted back into the blastema of a regenerating newt limb (Lo *et al.,* 1993, Kumar *et al.,* 2000) Thus, it is unlikely that each cell type requires a unique factor or set of factors to initiate its particular dedifferentiation cascade. A more parsimonious explanation is that the same factor initiates the dedifferentiation of all cell types However, the molecular and cellular mechanisms that govern epimorphic regeneration still remain incompletely defined

In the early stages of epimorphic regeneration in amphibians, cells migrate into the center of the blastema and make contacts, allowing cell-cell interactions to happen (Gardiner *et al.,*

1986) It is possible that after amputation at a particular proximal-distal (PD) location, cells arise with the appropriate level-specific identity and then generate more distal identities by successive local interactions (Gardiner *et al.,* 1995, French *et al*, 1976, Bryant *et al.,* 1981, Bryant and Gardiner, 1992, Bryant, *et al.,* 1993) The early dedifferentiation stage is exposed to a variety of signals from the wound epidermis and from the general injury response with retinoic acid (RA) (Viviano *et al.,* 1995), Hedgehog protein (Riddle *et al.,* 1993), and FGF (Boilly *et al.,* 1991) being some possible candidates, although the evidence for axial variation in expression in each case is either incomplete or absent Retinoic acid (RA) is known to respecify the PD axis during limb regeneration (Niazi and Saxena, 1978) and under certain circumstances to respecify the dorsoventral and anteroposterior axes (Kim and Stocum, 1986), furthermore, it can switch the identity of a tail blastema in some anuran tadpoles so that it gives rise to limbs (Mohanty-Hejmadi, 1993). Similarly, although the precise relationship between Hox gene expression and positional identity is not understood either for limb development or regeneration, the expression of HoxA9 and A13 in the mesenchyme is an important indication of local specification as early as 1 to 2 days after amputation (Gardiner *et al.,* 1995).

Regeneration is a complex phenomenon that requires input from a variety of factors like hormones, neural peptides, cytokines, growth factors, etc. Neural influence on epimorphic regeneration has been widely studied The proliferation of the *Salamander* limb regeneration blastema is dependent on the presence of nerves Singer (1952) demonstrated that a minimum number of nerve fibers must be present for regeneration to take place It is thought that the neurons release mitosis-stimulating factors that increase the proliferation of the blastema cells (Singer and Caston, 1972, Mescher and Tassava, 1975) There are several candidates for these neural-derived mitotic factors, and each may be important Glial growth factor (GGF), known to be produced by newt neural cells, is present in the blastema, and is lost upon denervation When this peptide is added to a denervated blastema, the mitotically arrested cells are able to divide again (Brockes and Kinter, 1986). A fibroblast growth factor may also be involved. FGFs infused into denervated blastemas are able to restore mitosis (Mescher and Gospodarowicz, 1979) Another important neural agent necessary for cell cycling is transferrin, an iron-transport protein that is necessary for mitosis in all dividing cells (since ribonucleotide reductase, the rate-limiting enzyme of DNA synthesis, requires a ferric ion in its active site) When a hindlimb is severed, the sciatic nerve transports transferrin along the axon and releases large quantities of this protein into the blastema (Munaim and Mescher, 1986; Mescher, 1992) Neural extracts and transferrin are both able to stimulate cell division in denervated limbs, and chelation of ferric ions from a neural extract abolishes its mitotic activity (Munaim and Mescher, 1986, Albert and Boilly, 1988) The relationship between nervous system and regeneration was established long back since 1940's, by a

number of workers (Singer, 1946, 1947, 1952; Butler and Schotte, 1941, 1949; Schotte and Butler, 1944; Van Stone, 1955, 1957, 1964). Kamrin and Singer (1955), Simpson (1964, 1965), and Cox (1969) showed nervous participation in the lizard tail regeneration. During early phases of tail regeneration in *Mabuya Carinata*, Acetylcholinesterase and Butyrlcholinesterase (AChE and BuChE) are negligible indicating that nervous stimulation is not essential for the initiation and formation of the early regenerates. However, activity of both these enzymes is higher during later phases of regeneration, suggesting their possible role in histo-differentiation of various tissues (Radhakrishnan, 1972). Though, a coordinated activity of all the factors, in time and space, is essential for truthful regeneration of the lost appendage, the maintenance of each stage is also mandatory to allow the regenerate to enter the next stage of regeneration uninterrupted. However, there are many by-products of the metabolic pathways that can interfere with the biological processes, the most deleterious of them being reactive oxygen species (ROS).

In normal metabolic processes, partially reduced and highly reactive metabolites are formed during electron transfer reactions, which include superoxide anion ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2). However, oxygen free radicals are continuously produced during aerobic metabolism (Halliwell, 1984), cells are protected from the toxic effects of these ROS due to antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and small molecules such as glutathione. During early events of epimorphic regeneration, there are series of metabolic processes that happen at a faster pace, hence these antioxidant enzymes and molecules are of great significance in protecting the cells from potential damage and maintaining the status quo. By and large excess ROS are detrimental to wound healing (Weiss, 1989) causing cell membrane and DNA damage, hence their detoxification is an essential step for successful healing of the wound. Further, recent studies have shown that ROS are not only harmful to the cells, but are also involved in the cell signaling events, when present at optimum levels (Finkel *et al.,* 1998; Pryor *et al.,* 1991). Furthermore, after healing of the wound during epimorphic regeneration, the cells of the AEC divide repeatedly to contribute to the formation of blastema.

The proliferation of blastema cells requires a number of growth factors from the wound epidermis and the nerves, which regenerate into the tip of the amputated limb (Nye et al, 2003). The wound epidermis is thickened into an apical epidermal cap (AEC), which is functionally equivalent to the AER of amniote embryonic limb buds and which is essential for the survival and proliferation of the subjacent mesenchymal cells. Growth factors expressed by the AEC and shown to promote blastema cell proliferation are Fgf-1, -2, and -8, while neural growth factors that have similar effects are Fgf-2 and Glial growth factor-2 (Ggf-2) (Brockes, 1984). The neurotransmitter substance P and the iron-binding protein transferrin,

as discussed earlier, are also neural factors that have a positive effect on blastema cell proliferation (Stocum, 2003) In addition, the blastema cells themselves express Fgf-10, which has been shown to be essential for limb regeneration in Xenopus (Yokoyama *et al*, 2001). Thus, growth factors are a group of substances that are identified as potent regulators of various cell functions during normal physiological as well as regenerative processes. 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;Kurup, 1996). Among the various growth factors involved in epimorphic regeneration, the role of Fibroblast Growth Factors (FGFs) has been studied at length in amphibians FGFs consist of a family of about twenty three members (FGF-1 to FGF-23), each consisting of a conserved core region of about 155 amino acids (Tanaka *et al.*, 2004). FGFs are small peptide growth factors with multiple biological functions which play significant roles in patterning, growth, and differentiation (Szebenyi and Fallon, 1999).

Several studies carried out by investigators on amphibian regeneration have established that fibroblast growth factor-2 (FGF-2) or basic FGF is one of the vital molecules to appear immediately after amputation at the site of autotomy, and is required for the early phases of epimorphic regeneration (Gardiner and Bryant, 1996) Some studies in amphibians have shown that FGF-2 can induce progression of epimorphic regeneration in a denervated stump, which otherwise would never regenerate (Mullen *et al.*, 1996), and thus FGF-2 is also required for the maintenance of the blastema. The stages of tail regeneration in lizards are similar to those in amphibians (Iten and Bryant, 1976), and hence, the present study was designed to investigate whether FGF-2 plays similar roles in epimorphic regeneration in reptiles as well

The first chapter of the present study dealt with the influence of FGF-2 on the progress of tail regeneration in the gekkonid lizard, *Hemidactylus flaviviridis*. To understand the stage specific influence of FGF-2 in tail regeneration, the experiments were carried out at three stages At first stage, FGF-2 was administered before autotomy was induced in the lizards. The subsequent observations on the progression of the tail regeneration in lizards revealed that the animals administered with FGF-2 showed faster healing of the wound as compared to that of the control animals. Thus, it appeared that the extraneously administered FGF-2 might have accelerated the healing process in the experimental animals Moreover, during epimorphic regeneration the process of wound healing is known to be accompanied by matrix reorganization, angiogenesis and the formation of a functional wound epithelium (WE) (Cohn *et. al.*, 1992). However, the formation of WE is a very critical event and is controlled by many factors including FGF-2. Once a functional WE is formed it releases necessary

signals for the further events in the process of tail regeneration Hence, in the present study the administration of FGF-2 before amputation was found to accelerate the formation of WE that is, in the animals treated with FGF-2 the WE appeared two-three days ahead as compared to control animals But in similar experiments with antiFGF-2, the healing of the wound as well as the formation of WE was delayed. Thus, the early healing of the wound could be attributed to the extraneous FGF-2 as several studies carried out during limb regeneration in amphibians demonstrate the presence of FGF-2 during the healing of the wound (Mullen *et al* , 1996; Zhang *et al.,* 2000, Ferretti *et al.,* 2001) Furthermore, several *in vitro* studies have also shown the involvement of FGF-2 in the wound healing process (Gibran *et al.,* 1994, Phillips *et al.,* 1993, Tsuboi *et al.,* 1992)

The formation of WE is followed by rapid cycles of cell division to accumulate a mass of pleuripotent cells called blastema In the present study, the early formation of blastema was observed in the animals administered with extraneous FGF-2, while it was significantly delayed in animals treated with antiFGF-2. Thus, FGF-2 might be involved in the increased proliferation of blastemal cells in experimental animals. Furthermore, the measurement of the rate of growth of the regenerates from 2-12 mm of growth, revealed that the rate of growth was significantly higher ($p \leq 0.01$) in FGF-2 treated lizards, and also from 12-24 mm ($p \leq 0\ 05$), when the animals were treated with FGF-2 before amputation Moreover, in the regenerates of the animals treated with FGF-2 the process of differentiation was found to be initiated earlier then the control animals, while treatment with antiFGF-2 delayed the onset of differentiation The animals administered with FGF-2 accumulated large number of blastemal cells faster, hence the differentiation started earlier, while in the antiFGF-2 treated animals the proliferative activities were hampered, as a result, the onset of differentiation was delayed Thus, the results of the current study undoubtedly showed the mitogenic role of FGF-2 during tail regeneration in lizards. Likewise, the role of FGF-2 in cell proliferation has been observed in various cell cultures as well as different animal models (DeHamer *et al.*, 1994, Folkman *et al* , 1989; O'Keefe, 1988, Dignass *et al* , 1994) Thus, it was evident from this experiment that extraneous administration of FGF-2 before amputation has a profound influence on the process of regeneration of tail in lizards which was further confirmed by the administering antiFGF-2

In the second set of experiments, the animals were injected with FGF-2 when they reached the WE stage Treatment with FGF-2 at WE stage significantly accelerated the formation of blastema as well as the onset of differentiation process. Conversely treatment with antiFGF-2 delayed both these processes. Hence, the administration of extraneous FGF-2 might be having positive influence in the proliferation of cells in the regenerate, which was also evident from the rate of growth of the regenerate. There was a significant increase in the

growth of regenerate from 2-12 mm and also from 12-24 mm in the animals receiving extraneous FGF-2, while animals treated with anti FGF-2 showed significant ($p \leq 0.05$) decrease in the rate of growth. The next set of experiment was conducted in which the animals were injected with FGF-2 and its antagonist when they reached the blastema stage. It was observed that there was only a marginal influence of extraneous FGF-2 on the process of tail regeneration, when administered at BL stage The rate of growth of regenerate was influenced positively only from 2-12 mm in the animals while from 12-24 mm the influence was only basal and comparable with that of controls Thus, FGF-2 might be involved in the cell proliferation during early phases of tail regeneration in *Hemidactylus flaviviridis*. Several studies support these results. For example, FGF-2 is known to stimulate proliferation of fibroblast, endothelial cells, and neuroectodermal cells (Baird and Walicke, 1989, Bennett and Schultz, 1993, Montesano, 1986) Similarly *in vitro* studies have also shown that FGF-2 promote proliferation of human bone marrow stromal cells (Ivan *et al.,* 1997; Martin *et al.,* 1997) Furthermore, FGF-2 is an exogenous regulator of smooth muscle cell migration and proliferation (Blaes and Allera, 1997).

In addition to being influencing the cellular processes, FGF-2 also influences the levels of macromolecules including DNA, RNA and protein. Therefore, a separate experiment was conducted to study the effect of FGF-2 on the levels of nucleic acids and protein in the regenerates (Chapter II) The administration of FGF-2 to the animals significantly increased ($p \leq 0.01$) the level of DNA in the regenerate at BL stage, suggesting high rate of replication and cell proliferation. Conversely treatment with antiFGF-2 decreased the DNA levels in the regenerate These observations were further strengthened by histofluroscence studies, which showed that the intensity of yellow fluorescence (DNA) was higher in the regenerates of the animals treated with FGF-2, unlike antiFGF-2 which showed lesser histofluorescence for DNA as compared to that of control animals Similarly, the RNA levels also showed a significant increase ($p \leq 0.01$) in the regenerate of FGF-2 treated animals. On the other hand, the levels of both the nucleic acids were significantly decreased ($p \leq 0.01$) in the regenerates of the animals treated with antiFGF-2 As far as protein levels in regenerate at BL stage are concerned it did not show any significant change in the FGF-2 treated animals, while the protein levels were significantly lower ($p \leq 0.01$) in the antiFGF-2 treated animals To supplement these observations the DNA/RNA ratios was calculated and it was found that this ratio was higher both in FGF-2 and antiFGF-2 treated animals at BL stage This observation indicated that the process of transcription was inhibited in both the groups. Thus, though FGF-2 increased the replication process, at the same time it negatively influenced the process of transcription Carreras *et al.,* (2001) has shown that basic fibroblast growth factor (FGF-2) decreases elastin gene transcription in confluent rat lung fibroblasts. Similarly the RNA/ Protein ratio was significantly higher in FGF-2 treated animals

at BL stage which indicated that there was a decrease in the process of translation. However, this ratio was found to be significantly low in the antiFGF-2 treated animals indicating an increase in the process of translation.

In addition, at differentiation stage the DNA levels in the regenerates of the animals treated with FGF-2 showed a significant increase Similarly, RNA and protein levels were also higher in the FGF-2 treated animals But, treatment with antiFGF-2 was found to decrease the levels of all the three macromolecules. Regarding DNA/RNA ratio, it was not altered much in antiFGF-2 treated animals, but FGF-2 treated animals showed a slight increase as compared to control animals This indicated that the process of transcription was slightly inhibited in FGF-2 treated animals while it was comparable in antiFGF-2 treated animals However, the process of translation was found to be elevated significantly in both the treatments indicating that during DF stage FGF-2 might not be involved in translational activities All these results suggested that during early phases of tail regeneration FGF-2 might be involved mainly in the cell division process occurring in the regenerate, either by promoting the synthesis of DNA or by interfering with the cell-cycle events as *in vitro* studies have shown that FGF-2 induces synthesis of DNA in different cell lines (Olwin *et al*, 1986; Imammura *et al.,* 1990). Thus, FGF-2 might be inducing quiescent cells to reenter the active cycles of cell division This role of FGF-2 might be traced back to its influence on cyclin D-CDK, complex which drives cells into mitotic phase This assumption finds supports from certain studies which say that FGF-2 increases the expression of Cyclin D and activates, cyclin D-CDK complex (Olson *et al.,* 2000; Rider and Jones, 1999) Along with the increase in the cell proliferation in the regenerate, there is also a need to sustain each of the stages, so that regenerates can step into the next stages without any hindrance The cellular processes such as cell division are accompanied with the formation of some by-products that might be harmful for progression into further events of the regeneration One such noxious byproduct is the formation of reactive oxygen species (ROS) that are formed during normal cellular processes (Halliwell, 1984) However, the cells possess antioxidant mechanisms to buffer the harmful effects of these oxidants or ROS and maintain the homeostasis Hence, a study was envisaged to understand the effects of FGF-2 on the antioxidant status (Chapter III)

The administration of FGF-2 was found to increase the activities of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) in blood and tissues (liver, Kidney, Intestine) at WE stage The high activity of SOD in the FGF-2 treated animals is a sign of detoxification of superoxide radicals formed during the healing process Similarly, higher activity of CAT might be an indication of high levels of H_2O_2, which might have been detoxified by CAT Thus, high CAT activity in blood and tissues might have aided in nullifying

the deleterious effect of H_2O_2. Further, the levels of reduced glutathione (GSH) were found to be elevated, while malondialdehyde (MDA) levels were quite lower in FGF-2 treated lizards. The increased levels of GSH showed that the cells were prevented from the damage caused by H_2O_2 whereas low levels of MDA indicated less peroxidation of membrane lipids, thus reducing the damage to the plasma membrane. And consequently, the cells are prevented from the negative effects of O_2^- radicals. Thus, FGF-2 was found to increase the activity of antioxidant defenses, thereby preventing the tissues from the harmful effects of ROS. Further, the activities of SOD and CAT in blood and tissues were found to be elevated at the BL stage too. The GSH levels were also found to be elevated accompanied by a decrease in the MDA levels. In addition, at DF stage, the activity of SOD was found to be elevated in blood and liver, while in kidney and intestine it did not show any significant change. Further, CAT activity was found to be elevated in blood, liver and kidney while in intestine it did not show any significant change. Besides, there were no observable changes in GSH and MDA levels in FGF-2 treated animals. Thus, it could be logical to surmise that FGF-2 might be involved prominently in the detoxification of ROS during early stages of tail regeneration in gekkonid lizard, while in the later stages FGF-2 might not play any significant role in this direction. This could be indicative of the fact that the production of ROS might be more in the early stages of tail regeneration, which was counteracted by antioxidant enzymes activated in part by FGF-2. There are ample studies which have shown that FGF-2 activates antioxidant enzymes (Yong-Fang and Yong-Jie, 2001; Mattson *et al.,* 1995; Hou *et al.,* 1997). Similarly, Hou *et al.,* (1997) have shown that FGF-2 increases the levels of GSH. In addition, FGF-2 is also known to decrease the levels of MDA in mice and rats (Hu and Wu, 2001). Moreover, in the present study, the results obtained from the treatment with FGF-2 were confirmed by treating the lizards with antiFGF-2. The treatment with antiFGF-2 was found to decrease the activity of SOD and CAT at the WE stage. Further, it was also observed that the GSH levels were lower but the MDA levels were comparatively high in antiFGF-2 treated animals as compared to that of control animals. Thus, in the animals receiving antiFGF-2 the tissues were not protected from the deleterious effects of ROS, during WE stage, instead the damage was pronounced due to the significant ($p \leq 0.01$) inhibition of the antioxidant enzymes. Similarly, at BL stage too, comparable results were found in the antiFGF-2 treated animals. However, during the DF stage, the activity of SOD and CAT was found to be decreased in blood and liver only, while in kidney and intestine there was no significant change noticed. The GSH levels remained unaffected during DF stage in antiFGF-2 treated animals, while MDA levels were significantly high ($p \leq 0.01$). From the results of the above study it could be concluded that FGF-2 might be participating, along with other factors, in the defense of tissue from the damage by ROS, during early stages of tail regeneration. However, FGF-2 might not be the only factor playing role in epimorphic regeneration, there could be several factors involved, EGF being one of them. Hence, a

separate experiment was conducted to study the role of EGF on tail regeneration in gekkonid lizard (Chapter IV).

To know the stage specific influence of EGF the animals were injected at three stages *viz.* before amputation, at WE stage and at BL stage The extraneous administration of EGF before amputation was leading to the early healing of the wound as compared to that of controls The blastema was also found getting formed faster in the EGF treated animals Further, these animals reached differentiation stage ahead of control animals The measurement of the tail lengths at different intervals revealed that the rate of growth was significantly higher in the treated animals as compared to control animals In the animals treated at WE stage, it was found that the blastema was formed earlier than control animals Even the rate of growth was found to be higher during the early phase of tail regeneration in treated animals. There was also an early onset of differentiation stage in experimental animals However, when injected at BL stage, the treated animals did not show any significant influence on the process of tail regeneration Thus, EGF, like FGF-2, might be involved in the early events of tail regeneration, *viz.* wound healing and cell proliferation There are reports which say that EGF participates in the healing of the wound in humans (Babul *et al.,* 2004, Hoppenreijs *et al.,* 1992). Moreover, *in vitro* studies have also confirmed the involvement of EGF in healing of the wounds in different cell cultures (Blay and Brown, 1985, Egan *et al.,* 2003) The process of regeneration in the early stages of tail regeneration was observed getting enhanced by supplementation with EGF Hence, EGF might also be playing definite role in proliferation of blastemal cells This may be supported by the *in vitro* studies carried out by several investigators in neuroprogenitor cells, where EGF stimulated their mitogenesis (Temple *et al.,* 1995, Vicario- Abejon *et al.,* 1995, Ghosh *et al.,* 1995)

The observed increase in the growth rate of the regenerate of EGF treated animals was also reflected in the nucleic acid levels in the regenerate There was a significant increase in the DNA levels in the regenerate of animals treated with EGF at blastemic stage. Similarly RNA levels were also comparatively higher in treated animals. The results of the DNA/ RNA ratio and RNA/Protein ratio indicated higher transcriptional and translational activities in the regenerates of experimental animals. During the differentiation stage, the DNA levels were significantly higher in experimental animals whereas RNA levels were not found to have any significant alteration However, protein levels were significantly ($P \leq 0.05$) higher in experimental group. The synthetic activities were found to be higher in EGF treated lizards This was also evident from histoflourescence studies (Figure 4.2 and 4.3) The observation that nucleic acid levels were elevated in regenerates of EGF treated animals finds supports from study by Murphy *et al.,* (1986) who reported that EGF stimulates DNA synthesis and cell proliferation in mammary epithelium in humans Similarly, many in vitro studies have

shown that EGF enhances the transcriptional and translational activities as well (Liu and Neufeld, 2004, Palmer *et al.,* 1995, Vescovi *et al.*, 1993)

In conclusion it might be hypothesized that both FGF-2 and EGF positively influence the process of tail regeneration in gekkonid lizard during the early stages of tail regeneration. However the finer mechanisms, by which these growth factors influence tail regeneration in *Hemidactylus flaviviridis*, need to be further elucidated