GENERAL CONSIDERATION

The regeneration of lost body parts and injured organs has captured the human imagination since the time of ancient Greeks Though, a large number of organisms are able to regenerate body parts, they have attracted relatively little research effort over the decades Broadly, the process of regeneration is known to be of two types *viz* morphallactic and epimorphic regeneration The process of morphallactic regeneration involves reorganization of the existing cells to form the lost structure, the resulting structure being comparatively smaller in size than the original However, the epimorphic regeneration involves the generation of new stem cells, either by proliferation of the existing stem cells or by dedifferentiation of adult cells, which redifferentiate to form the lost appendage which is of the similar size as that of the original. The epimorphic regeneration is prevalent in lower vertebrates and can be considered as a substitute for living a normal life for the animal. However, only few classes of lower vertebrates show true epimorphic regeneration like Urodeles and Anurans. More specifically, Urodele amphibians have the impressive ability to perfectly regenerate their tails throughout adulthood (Stocum, 2003) Usually, amputation to begin with results in wound healing, followed by the formation of a blastema of proliferating cells that go on to form the complete array of tissue types. It is still unknown how this diversity of cell types is reformed with such precision during regeneration. Although, the later events of patterning and redifferentiation during limb regeneration in amphibians are similar to those occurring during limb development, the early events, however, are quite unique to the process of epimorphic regeneration (Gardiner, 1997; Bryant *et al.,* 2002). Studies of gene expression have revealed striking differences between limb development and limb regeneration, particularly during the early stages of regeneration prior to blastema formation (Gardiner and Bryant, 1998). Survey of literature concerning epimorphic regeneration in amphibians suggests that the early events of epimorphic regeneration are controlled by several factors, including cytokines, growth factors, neural and hormonal factors. Moreover, there are many stages in the process of limb regeneration in amphibians, and each stage requires a set of factors to be expressed for the sustenance of the stages and progression into the next stage 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≤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≤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≤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≤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