INTRODUCTION

"...wide was the wound,
But suddenly with flesh filled up and healed."
—John Milton, Paradise Lost, VIII 467

WHAT IS REGENERATION?

Differentiated, somatic cells of animals may reinitiate development and produce a new organ to restore body parts that have been lost by injury or autotomy (self-amputation of body parts). This phenomenon is referred to as 'regeneration' (De Klerk, 2000).

HISTORY OF REGENERATION

Regeneration has always been a topic of interest in the history of science and medicine. The regeneration of organs and appendages is a theme found in the ancient Greek mythologies of the Hydra and of Prometheus, recounted by Homer and Hesiod (Dinsmore, 1996). The Second Labour of Hercules was to slay the nine-headed Hydra, feared for its remarkable power of regenerating two heads for every one sliced off. The Titan god Prometheus made the mistake of offending the supreme god, Zeus, by stealing fire from Olympus for the benefit of humankind. As punishment for this deed, Zeus had him chained to a rock, where each day his liver was eaten by an eagle, only to regenerate at night. This cycle of hepatectomy and regeneration would have been eternal had not Prometheus been rescued by Hercules. Accounts of regeneration of severed human arms and legs are also woven into the superstitions and descriptions of miracles in the middle ages (Goss, 1969).

Two thousand and four hundred years ago Aristotle first noticed that lizards could shed and regrow a lost tail. Later on regeneration became a focus of systematic scientific investigation in the 18th century. Abraham Trembly performed detailed experiments on the regeneration of hydra that made a deep impression on the biologists of that time, while Reaumer and Spallanzani had reported observations on the regeneration of limbs in crustaceans and newts, respectively (Dinsmore, 1991; Dinsmore, 1996). Studies on limb development and regeneration in the latter part of the 19th and the early part of the 20th

centuries made major contributions to the understanding of development. Prior to the 20th century, limb regeneration in amphibians and crustaceans was explained in the context of preformation. The limbs of these animals were presumed to contain multiple copies of preformed appendages, the growth of which was stimulated by amputation. In the early 20th century, regeneration was recognized as a regulative process that restored the whole from the remaining part. A major research aim of Thomas Hunt Morgan (1866 – 1945), before he turned to genetics, was to explain regeneration in terms of chemical and physical parameters.

Early studies of animal regeneration encompassed 3 basic foci: documenting that regeneration could occur, characterizing abiotic and biotic effects on the speed of regeneration e.g., Trembly (1740, cited in Emmel 1905) recorded that hydra heads could regenerate faster in warmer weather, and Spallanzani (1768, cited in Dinsmore 1996) showed that nutrient limitation could alter rates of regeneration, and exploring how regeneration occurs at the developmental/physiological level. By the 1960s, the focus of this work had shifted from an emphasis on the regenerative process itself to a broader concern for development in general.

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 (Goss, 1969; Wallace, 1981; for taxon-specific reviews, see- Crustaceans: Bliss, 1960; Juanes and Smith, 1995; Marsh and Theisen, 1999; Hopkins, 2001; Spiders: Vollrath, 1990; Insects: Needham, 1965; Bulliere and Bulliere, 1985; Echinoderms: Dubois and Ameye, 2001; Fishes: Wagner and Misof, 1992; Becerra *et al.*, 1996; Amphibians: Scadding, 1977, 1980; Tsonis, 1991; Brockes, 1997; Nye *et al.*, 2003; Lizards: Bellairs and Bryant, 1985).

REGENERATION IN DIFFERENT GROUPS OF ANIMALS

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 (e.g., poriferans, earthworms, and asteroids). However, the most common forms of regeneration–and those that are best characterized developmentally–involve appendages, such as regeneration of legs and tails in amphibian and lizards.

TYPES OF REGENERATION

In animals, based on cellular mechanisms, regeneration can be divided into two broad categories: a) morphallaxis and b) epimorphosis (Brockes, 1997).

a) ***Morphallaxis***: refers to the type of regeneration in which lost body parts are replaced by the remodelling (repatterning) of the remaining (existing) tissue. In this type of regeneration, little or no cellular proliferation (little or no growth) occurs during the regeneration process. A classic example of an organism that regenerates using this mechanism is the hydra. When, for example, a heavily irradiated Hydra is cut into two halves, cell divisions do not occur but both halves regenerate nevertheless, giving rise to two small Hydras both smaller than the parental Hydra. Once regeneration is completed, the two hydras continue to grow and reach the size of their original parent. This growth phase requires cellular proliferation but during the regenerative process very little cellular proliferation takes place.

b) ***Epimorphosis:*** In contrast to morphallaxis, epimorphosis requires active cellular proliferation prior to the replacement of the lost body part. Urodele amphibians or teleost fish have an astonishing capacity for regeneration. They may replace tails, limbs, jaws, and the lens of the eye. This involves growth of new tissue: this type of regeneration is denoted as epimorphic. Mammals can regenerate liver and can mend broken bones but the lost limbs are not replaced. In newts, after amputation, epidermal cells cover the wound to form a blastema. The cells of the blastema arise from beneath the wound epidermis, dedifferentiate, and start to divide. These cells become cartilage, muscle, and connective tissue. Limb regeneration is dependent upon the presence of nerve cells that produce the organogenic signal.

Epimorphosis can be further subdivided into **dedifferentiation-dependent** and **dedifferentiation-independent** subclasses. Planarians, which are flatworms, regenerate using a dedifferentiation-independent mechanism in which preexisting stem cells, known as neoblasts, begin to proliferate and migrate to the injured site in response to injury. These cells then form a mass of proliferating cells, known as the regeneration blastema that will later differentiate into the specialized cells that comprise the regenerated structure (Figure i). Most tissue regeneration in mammals also belongs to the dedifferentiation-independent subclass. For example, mammals can regenerate their muscle, bone, epithelia of the skin and gut, blood, and some neurons by activating preexisting stem cells or progenitor cells.

Certain vertebrates, such as the salamanders (newts and axolotls), regenerate lost body parts through dedifferentiation-dependent epimorphosis. In these cases, new stem cells or progenitor cells are created when differentiated cells reverse the normal developmental process and once again become precursor cells. These dedifferentiated cells then proliferate and later redifferentiate to form the regenerated structure or organ. Stem cells or progenitor cells are the common denominator for nearly all types of regeneration.

The other types of regeneration as described by Stoick-Cooper *et al.* (2007) are:

1) ***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).
2) ***Tissue regeneration***: repair of limited damage to an organ predominantly via restoration of only one cell type (e.g., skeletal muscle).

AUTOTOMY

A discussion of appendage/caudal regeneration is incomplete without first addressing the subject of autotomy. The term was originally introduced by Fredericq (1892) (originally called autotomie) and describes the reflex severance of an appendage without aid from any source other than from the severed appendage. An example would be the voluntary severance of a lizard tail to distract a predator that has not yet attacked.

There are 3 other closely related terms defined by various authors in the early 1900s (reviewed by Wood and Wood, 1932). "Autopasy" (also spelt autospasy) refers to a situation where an outside agent is responsible for the severance of an appendage at a preformed breakage plane (e.g., the loss of limb to a predator who has grasped it). "Autotilly," similar to autopasy, occurs when the animal itself removes an appendage with the assistance of its mouthparts or other legs. "Autophage" refers to the act of consuming a shed appendage usually, but not always, after severance from the remainder of the animal (e.g., some Lizards: Judd, 1955; Grant, 1957; Clark, 1969; and Eels: George, 1978). "Appendotomy" was introduced by Woodruff (1937) as an attempt to encompass all 4 of the previous definitions, but this term has not become popular. Despite the discrete situations described above, "autotomy," as it is used in current work simply mean appendage loss (with no implication of mechanism) and includes:

1. The reflexive loss of an appendage at a preformed breakage plane (e.g., the herpetology literature),
2. The loss of an appendage at a preformed breakage plane (e.g., the arthropod literature), or
3. The general loss of an appendage with no preformed breakage plane (e.g., the fish and amphibian literature) (McVean, 1975; Wilkie, 2001).

BASIC MECHANISM OF REGENERATION: A DIAGRAMMATIC REPRESENTATION

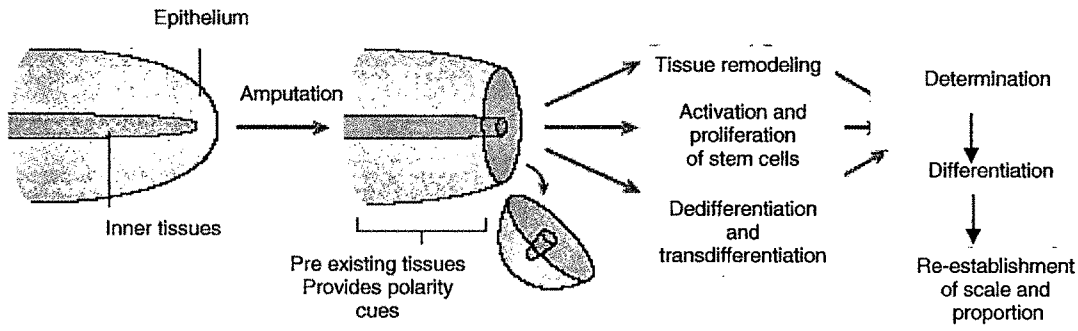

Figure i. Basic mechanisms of regeneration. After amputation, wound healing occurs. This is an injury response that is common to all animals, whether or not they can regenerate. After wound healing, if the resulting tissue stump is capable of regeneration, at least three processes can be activated, either independently or together. Hydra, for example, undergoes remodelling of pre-existing tissues to regenerate amputated parts. Lizards undergo both tissue remodelling and proliferation of resident adult somatic stem cells; in vertebrates, both stem-cell proliferation and the dedifferentiation or transdifferentiation of the cells that lie adjacent to the plane of amputation, takes place. The cells that respond to the stimulus of amputation eventually undergo determination and differentiation, resulting in new tissues that must then functionally integrate with and scale to the size of the pre-existing tissues (Adapted from Alvarado and Tsonis, 2006).

STAGES OF EPIMORPHIC REGENERATION

1) Wound epithelium or wound healing (WE) stage; 2) blastema (BL) stage followed by differentiation (DF) or morphogenetic phase.

WOUND EPITHELIUM (WE) STAGE

After amputation of the urodele limb or lizard tail, the surface of the wound is covered rapidly (within a matter of hours) with epidermal cells that migrate from the edge of the amputation surface, forming the "wound epidermis" (Figure i and ii) (for review, see Call *et al.*, 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, 1938; Chalkley, 1954; Bodemer and Everett, 1959; Hay *et al.*, 1962; Lo *et al.*, 1993; Kumar *et al.*, 2000). 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 *et al.*, 2005). The WE becomes a specialized structure (that some call the apical epithelial cap (AEC), which is distinct morphologically and in gene expression from the normal epithelium (Christensen and Tassava, 2000; Han *et al.*, 2003; Call *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 (Bryant *et al.*, 2002). Recent evidence shows that Wnt/β-catenin signalling is required for structural maturation of the WE in axolotls and frogs, but not for the earlier phase of epidermal migration after wounding (Kawakami *et al.*, 2006).

BLASTEMA FORMATION AND DIFFERENTIATION

Signals from the WE are thought to induce formation of the "regeneration blastema," which is comprised of progenitor cells that accumulate at the amputation plane, proliferate, and differentiate to direct regeneration at the distal tip of the regenerating limb/tail (for review, see Wallace, 1981; Tsonis, 1996) (Figure ii). The blastema is thought to be formed by differentiated cells that dedifferentiate upon amputation of the limb and/or by activation of resident stem cells (figure 2). It has been shown that differentiated cells of many tissues in the urodele limb stump dedifferentiate to give rise to the blastema. *In vitro* and *in vivo* evidence demonstrates that muscle tissue dedifferentiates from multinucleated myotubes (in culture) and myofibres (*in vivo*) to form mononucleated cells that proliferate and contribute to the blastema (Namenwirth, 1974; Lo *et al.*, 1993; Kumar *et al.*, 2000; Echeverri *et al.*,. 2001; Brockes and Kumar, 2002). Tanaka and Brockes (1998) that the blood clotting proteinase thrombin may act as an extracellular signal that induces this process, as it can indirectly induce S-phase re-entry in cultured newt myotubes. It is also known that 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 required for myotube cell cycle re-entry *in vitro* (Tanaka *et al.*, 1997; Kumar *et al.*, 2004; Alone *et al.*, 2005). Rab11 is required during Drosophila eye development which helps in the major vesicular transport of bio-molecules across the cells.

STEPS IN THE FORMATION OF BLASTEMA

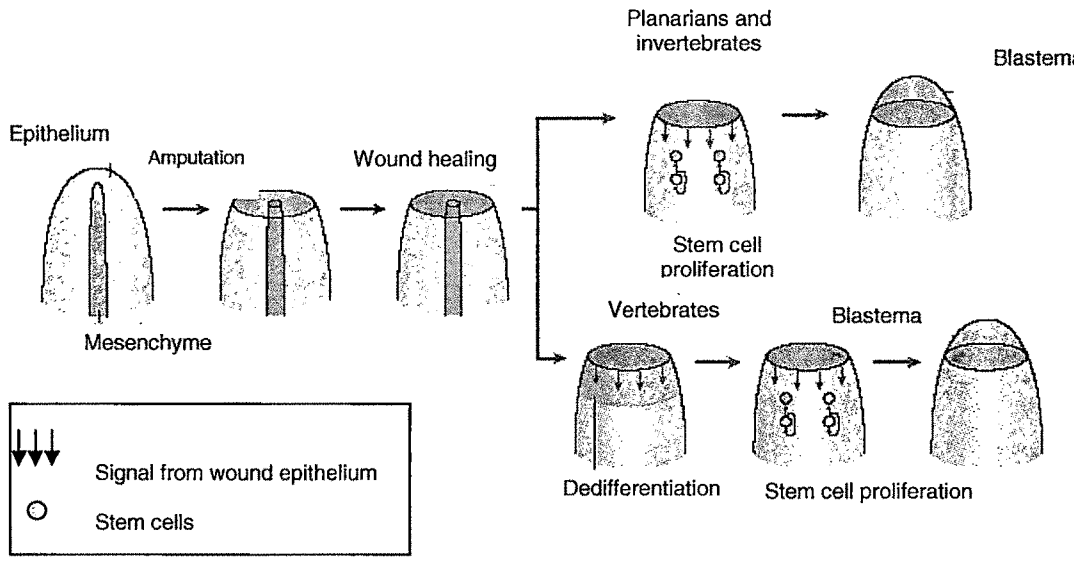

Figure ii. Basic steps in the formation of regeneration blastema in vertebrates and invertebrates. In vertebrates, there is evidence that both stem cells and cell-dedifferentiation processes have a role in blastema-mediated regeneration. In invertebrates such as planarians, stem-cell proliferation seems to have a pivotal role (Adapted from Alvarado and Tsonis, 2006).

POSSIBLE CUES FOR REGENERATION

The cellular and molecular mechanisms that govern epimorphic regeneration still remain incompletely defined. Many studies have found the key regulators of epimorphic regeneration. It is well known that limb regeneration requires innervation (for review, see Wallace, 1981). It has been suggested that axons secrete what have been called "neurotrophic factors" in the site of amputation and that these factors up-regulate genes important for the regenerative process. Fibroblast growth factor 2 (FGF2) may be one such factor (Mullen *et al.*, 1996). It is detected at the protein level in the WE and in nerves, and its expression decreases dramatically after denervation. Furthermore, FGF2-soaked beads can rescue regeneration in denervated regenerates (Mullen *et al.*, 1996). It has been recently

shown that innervation is required for maintenance of expression of genes (including *tbx5* and *prx1*) in the early blastema of the froglet, and for the initiation of expression of *msx1, fgf8, and fgf10* in the late blastema (Suzuki *et al.*, 2006). Fibroblast growth factor 8 (*fgf8*) and *fgf10* expression levels are also reduced in the denervated blastema of the axolotl (Christensen *et al.*, 2000), suggesting that the requirement of neuronal input for FGF expression is a conserved feature among species. 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 (Kostakopoulou *et al.*, 1996). FGF signalling is also essential for the early events in the development of the chick nervous system and mesoderm (Khot and Ghaskadbi, 2001) and also that FGF is one of the most important mesoderm induction factor in amphibians and chicks (Ghaskadbi, 1996).

Tail regeneration occurs in steps similar to limb regeneration and involves formation of a blastema (Stoick-Cooper *et al.*, 2007). The major signals regulating tail regeneration are bone morphogenetic protein (BMP) and Notch signalling (Beck *et al.*, 2003). Both the signals are required for cell proliferation although BMP pathway and is independent of Notch signalling (Stoick-Cooper *et al.*, 2007). Schnapp *et al.* (2005) studied that sonic hedgehog (*shh*) signalling besides pattering is required for proliferation of surrounding mesodermal tissue (Figure iii).

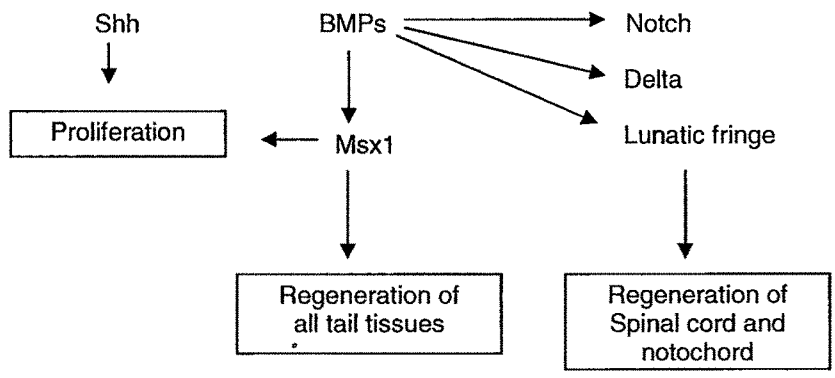

Figure iii. Major pathways involved in tail regeneration of urodele/lizard (Adapted from Alvarado and Tsnosis, 2006.

MAJOR PATHWAYS CONTROLLING CELL DIFFIERNTATION IN REGENERATION

Signalling pathway	Tail/Limb Regeneration
TGFB	Yes
Notch	Yes
Wingless	Yes
Hedgehog	Yes
JAK/STAT Unknown	Yes
EGF receptor	Yes
FGF receptor	Yes
Toll/NFκB Unknown	Yes

Until recently most of the attempts to identify a number of hypothesized regeneration signals have been unsuccessful. Carlson, (1970) had suggested that morphogenesis of some of the tissues depend upon secondary cues. One such cue is prostaglandin E_2 (PGE_2) signalling. Significant role of PGE_2 is known in cell proliferation, cell migration and differentiation. However, scanty reports are there suggesting the involvement of PGE_2 in regeneration. This might be due to the lack of an appropriate assay to identify and test the function of such signals. In the present work novel assays (for identifying the PGE_2 signals) which play a crucial role in reptilian tail regeneration are being worked out. Here, in the test system the production of PGE_2 was blocked by inhibiting the action of cyclooxygenase-2 (COX-2). The stage specific usage of COX inhibitors leads to identify the PGE_2 signals that control the steps of tail regeneration in lizards. It is known that PGE_2 signals are sent and received between the wound epidermis, nerves, and fibroblasts, and that they are necessary and sufficient to induce dedifferentiation and the formation of new structures (Murakami and Kudo , 2004; Shen, 2006).

PROSTAGLANDINS

Prostaglandins, thromboxanes and leukotrienes, collectively referred to as 'eicosanoids', are the cyclooxygenase (COX) and lipooxygenase metabolites of arachidonic acid (AA) and the oxygenation products of polysaturated long chain fatty acids (Figure iv). Discovery of eicosanoids (from Greek eicosa=twenty; for twenty carbon fatty acid derivatives), was initiated in 1930 (Burr *et al.,* 1930; Kurzrok and Keib, 1930; Euler, 1934).

Prostaglandins, potent bioactive lipid messengers derived from arachidonic acid, were first extracted from semen, prostate, and seminal vesicles by Goldblatt and Von Euler in the 1930s and shown to lower blood pressure and cause smooth muscle contraction. Bergstrom and colleagues purified the first prostaglandin isomers during the 1950s and 1960s, and in 1964, Van Dorp *et al.* and Bergström *et al.* independently identified AA, a 20-carbon tetraenoic fatty acid (C20:4ω6). Samuelsson linked these observations when they elucidated the structures of the "classical" prostaglandins and demonstrated that they were produced from an essential fatty acid, AA (Bergström *et al.,* 1964).

All prostaglandins exhibit roughly the same structure as all are oxygenated fatty acids composed of 20 carbon atoms and contain a cyclopentane ring, a C-13 > C-14 trans-double bond, and a hydroxyl group at C-15 (Figure vi). They were classified into types A to I, dependeing on the modifications of the cyclopentane ring. The abbreviations are commonly followed by an index (for instance PGE_2), which indicates the number of double bonds present in the various side chains attached to the cyclopentane ring. Based on the number of these double bonds, prostaglandins were classified into three series 1, 2, and 3.

Prostaglandins are formed by most cells of the body and act as autocrine and paracrine lipid mediators, signalling at or immediately adjacent to their site of synthesis. They are not only key mediators of inflammation and involved in apoptosis, cell differentiation and oncogenesis, but also play critical physiologic roles in tissue homeostasis and function. For example, gastric mucosal protective function, sleep induction and vascular smooth muscles contraction and relaxation are all dependent upon these compounds (Wallace, 1992; Funk, 2001; Muller, 2004). The actions of prostaglandins are summarized in Figure iv.

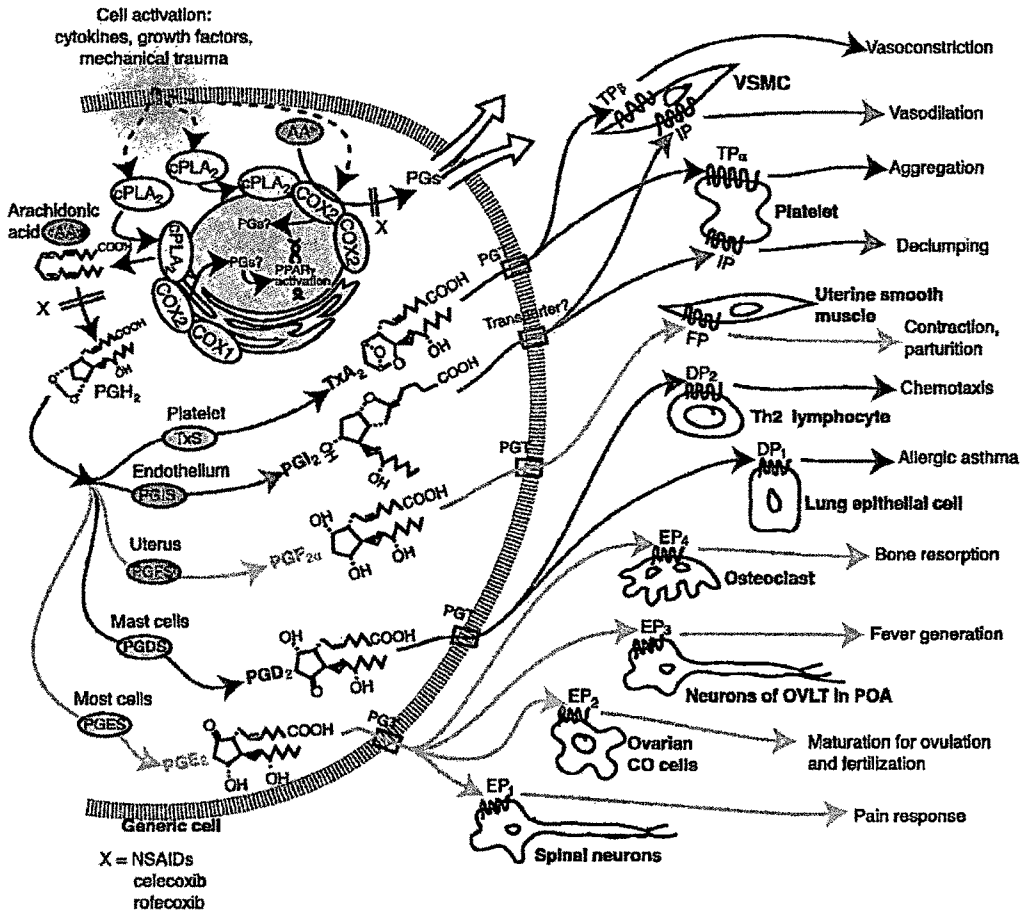

Figure iv. Prostaglandin synthesis and actions. Mechanical trauma, cytokines, growth factors, or various inflammatory stimuli activate cells, triggering signalling, including cytosolic phospholipase ($cPLA_2$) translocation to endoplasmatic reticulum and nuclear membranes, causing the release of arachidonic acid from membrane lipids and production of PGH_2 intermediate by COX-2 or COX-1. Heterogeneous family of PGH_2 metabolizing enzymes can form PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and TxA_2 (thromboxane). These prostaglandins may pass across a cell through a known prostaglandin transporter (PGT) to exert their actions on a family of prostaglandin receptors named EP1, EP2, EP3, EP4, DP1, DP2, FP, IP, TPα and TPβ (Funk, 2001) .

BIOLOGICAL FUNCTIONS OF PROSTAGLANDIN E_2

Prostaglandin E_2 is a major cyclooxygenase product in a number of physiological settings. It was first isolated and its structure determined in the 1960's (Samuelsson, 1963). Thereafter,

this prostaglandin was found in many different tissues. PGE_2 plays a protective role in maintaining the integrity of the gastric mucosa (Woo *et al.*,. 1986). The PGE_2 production in the kidney is critical for normal renal function by preserving renal blood flow and glomerular filtration rate in settings of physiological stress, modulating salt and water transport in the distal tubule, and stimulating renin release from the juxtaglomerular apparatus (Breyer and Breyer, 2001). PGE_2 was also shown to play a role in the maintenance of blood pressure, particularly in the setting of salt overload (Kennedy *et al.*, 1999). In certain instances, PGE_2 was observed to have multiple and apparently opposing functional effects. For example, PGE_2 elicits both smooth muscle relaxation and constriction (Walch *et al.*, 2001; Davis *et al.*, 2004). Complexity was also observed in modulation of the immune response by PGE_2; it was shown that PGE_2 regulates the function of many cell types including macrophages, dendritic cells, T and B lymphocytes leading to both pro- and anti-inflammatory effects (Hata and Breyer, 2004). PGE_2 signalling promotes tumour angiogenesis (Kurie and Dubois, 2001), increases cell proliferation and stimulates oncogenesis (Pai *et al.*, 2002). PGE_2 a ubiquitous inflammatory mediator, has been shown to regulate fibroblast proliferation, migration and synthesis and re-modelling of extracellular matrix (ECM). Prostaglandins have short half lives (seconds to minutes) make a special delivery system and also displays an extraordinary wide spectrum of biologic activity (Foegh and Ramwell, 2004).

The diverse effects of PGE_2 may be accounted for in part by the existence of four G-protein-coupled receptors, EP1, EP2, EP3, and EP4, and by heterogeneity in the coupling of these receptors to intracellular signal transduction pathways (Figure v). Synthesized PGE_2 is released by cells mainly through a prostaglandin transporter (PGT) (Schuster, 1998). The released PGE_2 then binds its receptors in the vicinity of the site of PGE_2 production. Of the four known EP receptors, EP3 and EP4 receptors bind PGE_2 with the highest affinity (Kd ~ 1 nM), whereas EP1 and EP2 receptors bind with lower affinity (Kd > 10 nM) (Abramovitz *et al.*, 2000). The EP1 receptor mediates PGE_2-induced elevation of free Ca^{2+} concentration, whereas the EP2 and EP4 receptors are coupled to Gs-type G protein and their activation leads to an increase in cyclic AMP (cAMP) levels. The signalling pathway of the EP3 receptor mediates inhibition of adenylate cyclase *via* Gi (Narumiya *et al.*, 1999).

Among the prostanoid receptors EP3 and EP4 receptors are widely distributed throughout the body. In contrast, the distribution of EP1 is restricted to several organs, such as the

kidney, lung, and stomach. The EP2 receptor is the least abundant of all the EP receptors. However, EP2 is effectively induced in the response to inflammatory stimuli (Narumiya *et al.*, 1999).

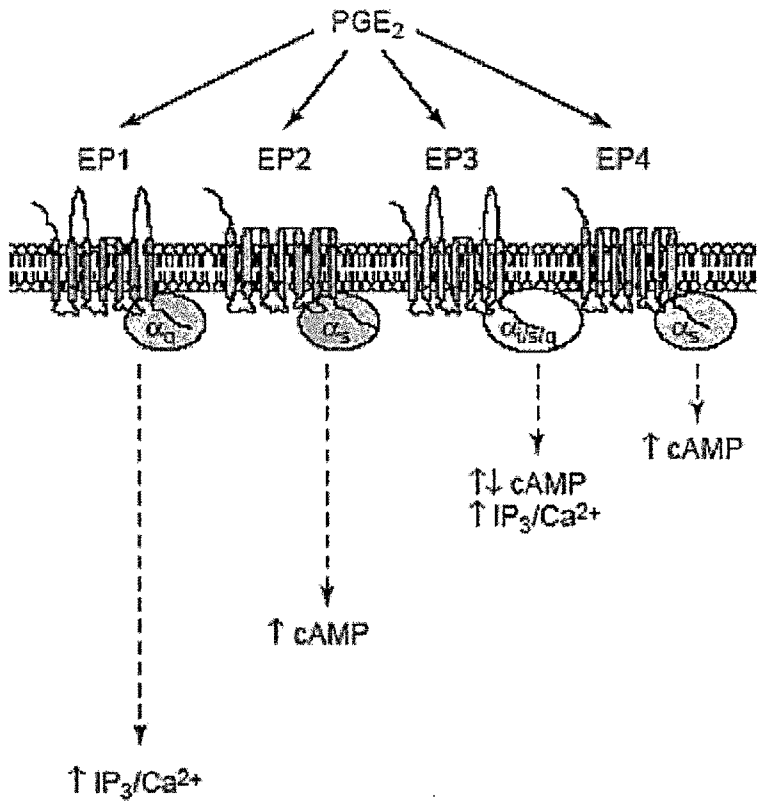

Figure v. Signal transduction of prostaglandin E_2 receptors. PGE_2 is actively transported out of the cell, where it exerts the effect by coupling to its heptahelical transmembrane receptors, EP1, EP2, EP3 and EP4 to activate second messengers, such as cAMP and inositol (1,4,5)-trisphosphate (IP3), and intracellular signalling cascades (Jabbour *et al.*, 2001).

ENZYMES INVOLVED IN PROSTAGLANDIN SYNTHESIS

Phospholipase A_2 enzymes

Arachidonic acid, the common precursor of eicosanoids, is stored at the sn-2 position of membrane glycerophospholipids, and is released by the hydrolytic action of phospholipase A_2 (PLA_2) enzymes (figure 4). Several PLA_2s were identified based on their nucleotide gene sequences. They were classified mainly into three groups: (i) cytosolic PLA_2 ($cPLA_2$), (ii) Ca^{2+}-dependent secretory PLA_2 ($sPLA_2$), and (iii) Ca^{2+}-independent intracellular PLA_2 ($iPLA_2$). Under stimulated conditions, Ca^{2+}-dependent cytosolic PLA_2s ($cPLA_2$s) and several secretory PLA_2s ($sPLA_2$s) are responsible for releasing AA from glycerol-based phospholipids to form PGs and related molecules (Jones *et al.*, 2003). Mitogen-activated protein kinases (MAPK), protein kinase A (PKA) and C (PKC) were shown to regulate PLA_2 activity (Pinelli *et al.*, 1999; Thomas *et al.*, 2000; Antonio *et al.*, 2002; Chakraborti, 2003). The upregulation of PLA_2 enzymes results in the production of a free pool of AA that is required for the synthesis of various eicosanoids.

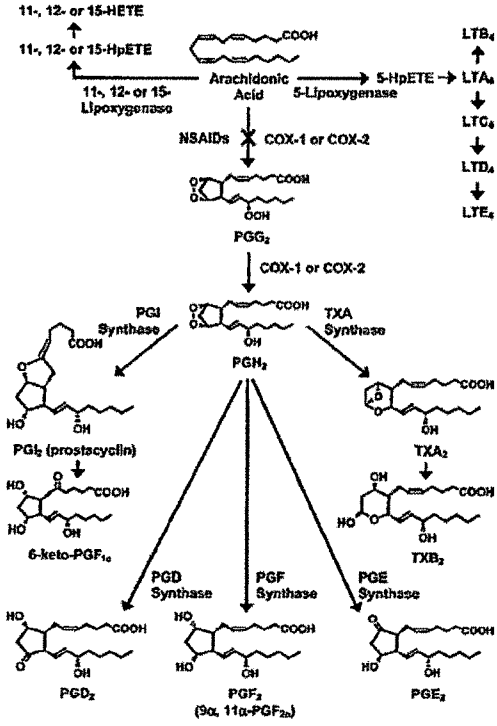

Figure vi. The arachidonic acid cascade. The fate of arachidonic acid in cells as it is metabolized by lipoxygenases to HETEs and hydroperoxyeicosatetraenoic acids (HPETEs) or by cyclooxygenases to prostaglandin H2 via the short-lived hydroperoxyl-containing intermediate prostaglandin G2. NSAIDs block the synthesis of prostaglandin G2. Prostaglandin H2 spontaneously rearranges or is enzymatically isomerized, oxidized, or reduced to yield bioactive prostaglandin isomers, some of which are shown. (Vane, 2002)

Cyclooxygenase enzymes

Arachidonic acid is metabolized to the unstable intermediate prostanoid, prostaglandin H synthase (PGH_2), by the action of COX enzymes. COX, also known as prostaglandin H synthase, is a heme-containing enzyme that catalyzes two sequential enzymatic reactions; first, the bis-oxygenation of AA leading to the production of PGG_2 (COX reaction) and second, reduction of 15-hydroperoxide of PGG_2 leading to the formation of PGH_2 (hydroperoxidase reaction) via separate active sites on the same enzyme (Smith *et al.*, 2000). Two COX isoforms, COX-1 and COX-2, are found in mammals. Both enzymes are encoded by separate genes on different chromosomes: COX-1 is located on chromosome 9, COX-2 on chromosome 1. COX-1 and COX-2 display ~ 60% sequence identity and both possess homodimeric structure. Both COX have a molecular weight of 72 kDa and are made up of a dimeric complex of two polypeptides, each of which requires one molecule of heme for maximal catalytic activity (Roth *et al.*, 1981; Brown and Dubois, 2005).

It is commonly considered that COX-1 is constitutively expressed in a wide variety of cells and plays a housekeeping role, whereas COX-2 is a stimulus-inducible enzyme that is implicated in pathological conditions, such as inflammation, pain, fever and cancer (Morita, 2002). A ten to eighteen fold increase in the expression of COX-2 occurs when stimulated by growth factors, tumour promoters and cytokines (Foegh and Ramwell, 2004). In general, COX-1 is more enriched in the Golgi, endoplasmic reticulum (ER) than in the perinuclear envelope and COX-2 is located predominantly in the perinuclear envelope. The promoter structure of the COX-1 gene suggests that specificity protein-1 (Sp1), activator protein-2 (AP-2), and nuclear factor for interleukin-6 (NF-IL-6) are involved in the regulation of COX-1 gene expression. The two Sp1 sites contribute to constitutive expression of COX-1 (Xu *et al.*, 1997). The promoter region of the COX-2 gene reveals a typical feature of the immediate early genes which contains various transcription elements, such as NF-IL-6, AP-2, Sp1, NF-kB and cAMP response element (CRE). Three mitogen-activated protein kinase (MAPK) cascades: (i) extracellular signal-regulated kinase1/2 (ERK1/2), (ii) c-jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK), (iii) p38 (Simmons *et al.*, 2004) contribute to the induction of COX-2 gene concertedly or independently. The nuclear receptor, peroxisome proliferator-activated receptor (PPAR) γ has also been shown to regulate the expression of COX-2 (Meade *et al.*, 1999; Inoue *et al.*, 2000; Straus *et al.*, 2000).

Cyclooxygenase-2 induction is associated with various premalignant and malignant lesions of epithelial and nonepithelial origin. COX-2 dependent biological responses had received much attention in the past few years because numerous pharmacological, biological and genetic studies have suggested that this inducible COX isoenzyme is involved in inflammation and cancer (Murakami *et al.*, 2000), in a view of the fact that PGE_2 is produced via COX-2 dependent pathway (Murakami and Kudo, 2004). COX-2 expression was inhibited by usage of selective and nonselective inhibitor of COX-2, NSAIDs, have been known to inhibit cancer formation in rodent models of colorectal cancer since the 1980s, and the development of selective COX-2 inhibitors has shown equal promise in rodent models and in several models of colorectal cancer (Brown and Dubois, 2005). Together these studies underline the important role of COX-2 induced PGE_2 in the initiation and promotion of cells during regeneration.

In 2002, Chandrasekharan and colleagues reported the isolation of a splice-variant of COX-1 mRNA found in highest concentrations in the cerebral cortex and heart of the dog, which they termed "COX-3". COX-3 is made from the COX-1 gene but retains intron 1. This intron contains an open reading frame that introduces an insertion of 30–34 amino acids, depending on species. COX-3 was postulated to be a 65-kDa protein. COX-3 mRNA is mainly expressed in cerebral cortex and heart and exhibits glycosylation-dependent COX activity. Comparison of COX-3 with COX-1 and COX-2 demonstrates that this enzyme is selectively inhibited by analgesic/antipyretic drugs, such as acetaminophen and phenacetin (Murakami *et al.*, 2000).

Prostaglandin E synthases

The final step of the PGE_2-synthesizing cascade is a nonoxidative rearrangement of the COX product, PGH_2, into PGE_2 (figure 4). Although this isomerization may also occur nonenzymatically, living cells produce PGE_2 via a catalytic reaction, which is attributed to several discovered proteins: cytosolic prostaglandin E synthase, microsomal prostaglandin E synthase-1 and microsomal prostaglandin E synthase-2 (Watanabe *et al.*, 1999; Jakobsson *et al.*, 2000; Tanioka *et al.*, 2000).

MODE OF ACTION OF COX INHIBTORS

Non-steroidal anti-inflammatory drugs, usually abbreviated to NSAIDs, are drugs with analgesic, antipyretic and anti-inflammatory effects - they reduce pain, fever and

inflammation. The term "non-steroidal" is used to distinguish these drugs from steroids, which (among a broad range of other effects) have a similar eicosanoid-depressing, anti-inflammatory action. NSAIDs work was that they inhibited the constitutive enzyme COX, decreasing prostaglandin synthesis, which in turn reduced the pain-sensitizing effect of the prostaglandins.

Generally, the classification NSAID is applied to drugs that inhibit one or more steps in the metabolism of arachidonic acid (AA). Unlike corticosteroids, which inhibit numerous pathways, NSAID act primarily to reduce the biosynthesis of prostaglandins (PG) by inhibiting cyclooxygenase (COX). In general, NSAID do not inhibit lipoxygenase (and hence leukotriene) formation, or the formation of other inflammatory mediators, although tepoxalin, a recently introduced NSAID does inhibit lipoxygenase

Cyclooxygenase is the main pharmacological target for NSAIDs. The observation that NSAIDs reduce or prevent the production of PGs by direct inhibition of COX enzymes was first reported by Vane in 1971. At present, NSAIDs are among the most widely prescribed class of pharmaceutical agents worldwide, having broad clinical utility in treating pain, fever and inflammation (Gilory *et al.*, 2001). The most popular NSAID, aspirin, is also sought as a potentially viable option in the prevention of sporadic colon cancer and neurodegenerative disorders (Applenton *et al.,* 1995). Most of these drugs are well absorbed, and food doesn't substantially change their bioavailability. Most of the NSAIDs are highly metabolized, some by phase I followed by phase II mechanisms. The introduction of the cyclooxgenase-2–inhibitory NSAIDs (Coxibs), selective inhibitors of cyclooxygenase (COX)-2, has resulted in large quantities of safety and efficacy data.

Skeletal muscle satellite cells play an important role in muscle regeneration. Previous work has suggested that nonsteroidal anti-inflammatory drugs may inhibit their activity. Various studies have demonstrated that the cultured skeletal muscle satellite cells from 9-month-old Sprague-Dawley rats when exposed to naproxen sodium (a nonselective cyclooxygenase inhibitor), NS-398 (a selective cyclooxygenase-2 inhibitor), and SC-560 (a selective cyclooxygenase-1 inhibitor) for 96hr resulted in decreased satellite cell proliferation (with Cyclooxygenase-2 inhibition alone). Inhibition of both cyclooxygenase-1 and cyclooxygenase-2 resulted in decreased satellite cell differentiation and fusion. This study suggests that the cyclooxygenase enzymes appear to play an important part in satellite cell

proliferation, differentiation, and fusion and that nonsteroidal anti-inflammatory medication may have an adverse effect on muscle regeneration following injury. Therefore, in the present experiment set up selective and non-selective COX-2 inhibitors were used. Nonselective COX inhibitors (brand name colosrpin) and selective COX-2 inhibitor (celecoxib and etoricoxib) were used in the present study to elucidate the role of prostaglandin pathway in reptilian regeneration.

LIZARD AS A MODEL OF REGENERATION

The basic criteria for selecting a suitable model system for study on regeneration are: 1) the animal should be one of the simplest in which regeneration is patently manifested and 2) the organism should be easy to handle and readily available through out the year. Among several animals considered, Northern House Gecko *Hemidactylus flaviviridis* Rupell fulfilled these criteria best, as they are known to display robust regenerative capacities. Further, being phylogentically closer to mammals (with its genome much closer to mammals with fewer gaps in evolution), the extrapolateion of the results to a mammalian system will be more meaningful.

Indeed, there exists plenty of scientific literature reporting experimentation with lizards (Clause and Capaldi, 2006). At our department several aspects of regenerations have been explored in lizards. Some basic studies on regeneration have been carried out on the gekkonind (*Hemidactylus flaviviridis, Hemidactylus brookie*), agmaid (*Calotes versicolor*) and scincid (*Mabuya carinata*) lizards. These studies have proved that there are metabolic and biochemical alterations in the tail during regeneration (Shah and Chacko, 1967, 1972; Shah and Ramchandran, 1970, 1972, 1973, 1974, 1975, 1976; Hiradhar, 1972, Shah and Hiradhar 1974, Ramchandran *et al.*, 1975; Kumar *et al.*, 1993; Kumar and Pilo, 1994; Pilo and Kumar, 1995). Moreover, the histological changes occurring during the caudal regeneration were also elucidated (Shah and Chacko, 1972; Kumar and Pilo, 1994). In the recent past, attempts were also made to understand the role of growth factors on tail regeneration. The results however, were contrasting wherein Epidermal Growth Factor (EGF) acted as a promoter for regeneration (Pilo and Suresh, 1994) while Tumour Necrosis Factor (TNF) and Nerve Growth Factor (NGF) were found impairing the process of regeneration (Kurup, 1997). A recent work was done to identify the role of neural peptides (FGF2) in lizard tail regeneration and a positive influence of growth factors was observed in growth of regenerate after induced autotomy (Yadav, 2005). Daniels *et al.* (2003)

investigated the process of lymphangiogenesis in regenerating tail of lizard with a view to develop possible treatments for human lymphoedema. Therefore, in the present study an attempt was made to untangle some of the important aspect of regeneration which will be helpful in designing regenerative medicine. Thus, lizard could be one of the potential and useful models for cellular and molecular basis of regeneration. Extensive work has been carried out in order to elucidate the underlying mechanism of regeneration but little attention has been focused towards the endogenous mediators which may be involved in resolution of inflammation and proliferation episode of regeneration.

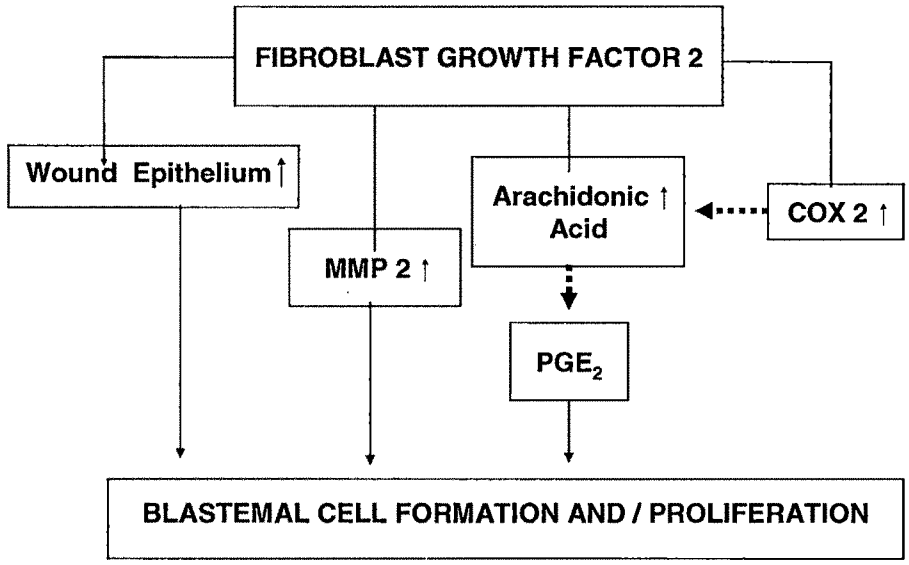

Figure vii: Schematic Representation of a Hypothetic Pathway Controlling Epimorphic Regeneration in Lizards

In one of the recent experiments carried out in our laboratory it was found that Fibroblast Growth Factor 2 (FGF2) plays a vital role in the process of caudal regeneration at least during the early phases (Yadav, 2005). The follow up studies were undertaken in which, the signalling pathway of FGF2 was implicated. FGF2 exerts pleiotropic effects during regeneration that include angiogenesis and matrix remodelling. FGF2 is one of the potent

stimulator of cell proliferation and migration which is also known to trigger arachidonic acid (AA) metabolism (Bikfalvi *et al.*, 1997). The later is an important substrate in lipoxyegenase (LOX) and cyclooxygenase (COX) pathways (Figure vii). Prostaglandins are required for cellular proliferation and they are also involved in various stages of myogenesis during regeneration (Rudnick *et al.*, 2001; Brenda *et al.*, 2004).

AIM OF THE STUDY

In order to understand the role of PGE_2 in epimorphic regeneration, in the current study, the upstream modulators of PGE_2 and the cyclooxygenase isoforms were targeted. The animals were administrated with the traditional NSAIDs as well as COX-2 specific inhibitor and effect of these drugs on various phases of regeneration was evaluated morphometrically. The study therefore, reveals the morphological changes occurring due to the exogenous blockage of all isoforms of COX and also COX-2 alone. Since both the treatments resulted in statistically identical outcome in achieving various milestones of regeneration, it was concluded that COX-2 plays a key role in hampering regeneration. Hence, further studies were carried out with COX-2 specific inhibitor (Etoricoxib) only.

Further, early events of epimorphic regeneration are characterized by heightened cellular activities with simultaneous increase in metabolic processes (Bryant *et al.*, 2002). During these metabolic processes highly reactive metabolic by-products, like super oxide anion ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2) collectively termed as reactive oxygen species (ROS), are formed. Previous studies have demonstrated that receptor binding of numerous peptide growth factors and AA metabolism stimulate ROS production and that this oxidant burst is required for certain aspects of downstream signalling (Droge, 2002). Moreover, FGF2 is also known to elicit antioxidant enzymes in the system (Yong-Fang and Young-Jie, 2001). The biological system is being protected from the toxic effects of these ROS due to the endogenous production of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) etc. By and large excess production of ROS causes cytotoxicity and cell death (Droge, 2002). It is known that superoxide can serve as a growth signal in different cells via rac/ras-NAD(P)H oxidase-MAPK signalling pathway. It was also noticed that over or under expression of ROS exert a growth stimulation effect on wide variety of cells and organisms. Therefore, it was worth quantifying the antioxidant enzymes at different phases

of caudal regeneration while the PGE_2, which is considered as another source of ROS production, synthesis is blocked (Kawaguchi *et al.*, 1995; Majima *et al.*, 2000).

Regeneration comprises several overlapping cellular processes, including inflammation and myogenesis. The wound healing stage during lizard tail regeneration comprises many events like apoptosis of deformed and damaged cells, extracellular matrix degradation with help of the proteases viz. MMP-2 and MMP-9 and proliferation of cells to heal the wound. The phenomena of regeneration consist of a lot of protein activities and proteases mediated matrix digestion. The alteration in protein expression was appraised through normal SDS PAGE. Trappe *et al.* (2001) showed that reduction in PGE_2 synthesis leads to reduction in protein synthesis. The widely studied matrix metalloproteinase in epimorphic regeneration are MMP-2 and MMP-9. PGE_2 is known to regulate the expression of MMP-2 and MMP-9. Therefore, gelatin zymography was used to determine the effects of inhibition of PGE_2 on the expression of these MMP enzymes. Further, to confirm the presence of MMP-2 and MMP-9 in the regenerate, histochemical localization was performed.

The epimorphosis in lizard nevertheless occurs with an array of sequences starting with wound closure and ending with the appearance of a fully grown regenerate. The formation of blastema is achieved by the recruitment of already existing stem cell or by the dedifferentiation of adult stump cells. Similar to amphibians, lizards also possibly generate these pleuripotent cells by the process of dedifferentiation (Kumar *et al.*, 2000). This process involves tissue remoulding, cell migration and proliferation. In order to scrutinize the role of COX-2 induced PGE_2 in cell proliferation of lizard tail, the mitotic index was studied by bromodeoxyuridine (BrdU) incorporation and subsequent measurement of histofluorescence at various stages of regeneration. Moreover, experiments were also conducted to document the histological changes occurring due to impaired PGE_2 synthesis during every stage, in order to have an additive support for the morphological amendments in the regenerate. Synthesis of prostaglandins during inflammation has a critical role in vascular cell proliferation (angiogenesis). Thus, the immunohistochemisty of vascular endothelial growth factor (VEGF), one of the important markers for angiogenesis, was studied. Also, a treatment induced alteration in myogenic proteins (desmin and myosin) was studied using histofluorescence microscopy, for the further evaluation of a significant role of prostaglandin in regeneration.

Lastly, in order to study the present objective, the role of COX-2 induced PGE_2 in caudal regeneration, COX- 2, an upstream modulator of PGE_2 was inhibited in Northern House Gecko, *Hemidactylus flaviviridis* and the following processes of epimorphosis were evaluated:

- The initiation and progression of tail regeneration - a morphometric analysis.
- Antioxidant status during various stages of tail regeneration in *Hemidactylus flaviviridis* when subjected to specific-COX-2 inhibitor.
- Regulation of stage specific expression of proteins and proteases.
- Role of prostaglandin E_2 in different cellular activities during epimorphic regeneration *viz.* cell proliferation, angiogenesis and myogenesis.

MATERIALS AND METHODS

MATERIALS

ANIMAL AND MAINTENANCE

Adult wall lizards Northern House Gecko, *Hemidactylus flaviviridis* Rüppell, 1835, (both the sexes) with intact tails weighing 10 ± 2g, collected from pristine natural habitat, were procured from local animal dealer. They were housed in well ventilated wooden cages of 45 x 30 x 60 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. Room temperature was maintained at 30 ± 2°C, as this temperature is necessary to produce optimum tail growth in lizards. All animals were screened for parasitic infestation and the healthy ones were acclimated for a week before the commencement of experiment. The animals were fed with in-house reared cockroach nymphs twice a week and purified water was given daily, *ad libitum.*

METHOD FOR INDUCED AUTOTOMY

Autotomy was induced by exerting mild thumb pressure on the normal intact tail three segments away from the vent. All experimental protocols were approved by IAEC (Institutional Animal Ethics Committee) according to CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India. The experiments were performed in accordance with the guidelines of the Animal Care. Unless otherwise stated, the sample size per group was at least five.

ESTABLISHMENT OF DRUGS DOSAGES

Animals were given *in loco* (IL) injections. The drugs dosages were selected from the reference data for the drugs. In case of colosprin the oral LD_{50} values for mouse is: 250 mg/kg; for rat: 200 mg/kg; and for rabbit: 1010 mg/kg body weight. The dose for celecoxib was selected by referring to the Area Under the plasma concentration time Curve (AUC). The dose for etoricoxib was decided from the reported oral LD_{50} value which was 1499 mg/kg body weight for both female mice and rats. Based on the above reports and also following a dose range study the following dosages were selected.

SOLUBILITY OF DRUGS

The drugs were soluble in alkaline pH. Therefore all the drugs were prepared in Tris Buffer pH 8.8. Drugs were always made fresh before use.

(i) Colosprin: *In Loco* (IL.) administration: 100 mg/kg body weight for high dose and 50 mg/kg body weight for low dose was prepared in Tris Buffer (pH 8.8), immediately before use.

(ii) Celecoxib: *In Loco* (IL.) administration: 100 mg/kg body weight for high dose and 50 mg/kg body weight for low dose was prepared in Tris Buffer (pH 8.8), immediately before use.

(iii) Etoricoxib: *In Loco* (IL.) administration: 100 mg/kg body weight for high dose and 25 mg/kg body weight for low dose was prepared in Tris Buffer (pH 8.8), immediately before use.

All the drugs *viz.* colosprin, celecoxib and etoricoxib were gifted from Sun Pharma Co., Vadodara, India. All other chemicals were of AR grade and procured from Qualigens fine chemicals, Mumbai, INDIA. , Bangalore Genei, HiMedia, SRL and the antibodies were procured from Sigma St Louis, USA and R & D Systems, Inc. Minneapolis, USA.

METHODS

EXPERIMENTAL PROTOCOL

The animals were treated with the drugs four days prior to autotomy and the treatment was continued after autotomy till the differentiation stage. After amputation, the process of wound healing initiates, followed by the formation of wound epithelium. The wound epithelium appears as a smooth shining surface and was accompanied by the process of dedifferentiation. Blastemic stage is characterized by conical aggregation of cells called blastemal cells (approximately 1-2 mm in length from the stump), which have been formed as a result of dedifferentiation process. The blastema further grows in size and later on differentiates to replace the missing structures.

MORPHOMETRIC MEASUREMENTS OF TAIL GROWTH

The growth in the length of the tail regenerate was measured using a calibrated digital Vernier Calliper (Mitutoyo, Japan), at fixed intervals. The time taken to reach the various stages of tail regeneration, such as wound epithelium (WE), early blastema and differentiation were recorded (Figure 1.2, 1.3).

FOURIER TRANSFORM INFRARED SPECTOROSCOPY (FTIR)

Principle: FTIR is used to identify chemicals from spills, paints, polymers, coatings, drugs, and contaminants. FTIR is perhaps the most powerful tool for identifying types of chemical bonds (functional groups). The wavelength of light absorbed is characteristic of the chemical bond as can be seen in this annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. FTIR spectra of pure compounds are generally so unique that they are like a molecular "fingerprint". While organic compounds have very rich, detailed spectra, inorganic compounds are usually much simpler. For most common materials, the spectrum of an unknown can be identified by comparison to a library of known compounds. To check the presence of the drug in the animal model the above study was used.

SAMPLE PREPARATION

Samples for FTIR can be prepared in a number of ways. For liquid samples, the easiest is to place one drop of sample between two plates of sodium chloride (salt). Salt is transparent to infrared light. The drop forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) to form a very fine powder. This powder is then compressed into a thin pellet which can be analyzed. In addition KBr is also transparent in the IR. Alternatively, solid samples can be dissolved in a solvent such as methylene chloride, and the solution placed onto a single salt plate. The solvent is then evaporated off, leaving a thin film of the original material on the plate. This is called a cast film and is frequently used for polymer identification. Solutions can also be analyzed in a liquid cell. To check the presence of the drug etoricoxib in the tail tissue, following protocol was employed:

Tail is homogenized in 0.1 N HCl and then kept on the sonicator for half an hour. After that the sample was separated in a separating funnel by adding an equal amount of chloroform as the homogenate. The funnel was left undisturbed for 10 minutes and than the lower layer was extracted which contained the sample of interest that was to be analyzed in liquid cell. The

DRIFTS spectra of tissue etoricoxib were obtained from this organic tissue extract, after appropriate background subtraction, using an FTIR spectrometer (FTIR-8400, Perkin Elmer). About 0.5 to 1 ml of the sample was directly taken in sodium cell, and the sample was scanned from 4,000 to 400 cm^{-1}. The resultant peaks were compared with standard etoricoxib in different concentration.

BIOCHEMICAL ESTIMATION

BLOOD COLLECTION

Blood was collected in pre coated EDTA vials by puncturing the heart.

HEMOLYSATE PREPARATION

Blood was centrifuged at 3000rpm for 10mins. After centrifugation, plasma was separated and transferred to vials. The erythrocyte sediment was washed 3 times with 0.6 % saline. After each wash the samples were spun briefly for 3 min at 3000rpm. The separated plasma was replaced by an equal volume of distilled water. The sediment was then homogenized and used for various biochemical assays.

ANTIOXIDANT PARAMETERS

CATALASE (CAT) ESTIMATION [EC 1.11.1.6]

Catalase activity was estimated according to the method described by Sinha *et al.*, (1972)

Principle: Catalase exerts dual functions: Firstly decomposition of H_2O_2 to give H_2O and O_2 [Catalytic activity, EQ (1)] and secondly oxidation of H donors, eg, methanol, ethanol, formic acid, phenol, with the composition of 1 mol of peroxide [peroxide activity, EQ (2)].

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2 \qquad (1)$$

$$ROOH + AH_2 \xrightarrow{CAT} 2H_2O + ROH + A \qquad (2)$$

The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate, when heated in the presence of H_2O_2 with the formation of perchromic acid as an unstable intermediate. The chromic acetate thus produced is measured colorimetrically at 620 nm. Since dichromate does not absorb light at this wavelength, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate. The catalase preparation is allowed to catalyze the breakdown of H_2O_2 for varying

periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically after heating the mixture.

Procedure: To 0.9 ml of phosphate buffer 0.1 ml of enzyme preparation and 0.4 ml of H_2O_2 were added. After 30 seconds and 60 seconds, 2.0 ml of dichromate reagent was added to terminate the reaction. The tubes were maintained in boiling water bath for 10 minutes and the colour developed was read at 620 nm.

Standards in the range of 2-10 μmoles were taken and processed as the test. The activity was expressed as μmole of H_2O_2 consumed/minute/mg protein.

LIPID PEROXIDATION LEVELS (LPO) ESTIMATION

It was estimated according to the method described by Beuge and Aust (1978).

Principle: Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) and gives thiobarbituric acid reactive substance (TBARS). TBARS gives a characteristic pink colour that can be measured colorimetrically at 532 nm.

Procedure: 1.0 ml of 4 % (w/v) tissue homogenate was added with 1.0 ml of TBA reagent in a test tube, the mouth of the test tube was plugged with cotton and the tubes were kept in boiling water bath for 20 minutes. The tubes were allowed to cool and centrifuged at 3000 rpm for 15 minutes. The clear supernatant was read at 532 nm. The calculation was done as per the slope obtained from the standard graph and the values were expressed as nmoles of MDA formed/mg tissue.

SUPEROXIDE DISMUTASE (SOD) ESTIMATION [EC 1.15.1.1]

It was estimated according to the method Marklund and Marklund (1974).

Principle: Pyragallol auto-oxidizes at pH 8.0. SOD inhibits this auto-oxidation of pyragallol in a rate limiting fashion. 50% inhibition of pyrogallol auto-oxidation is equivalent to 1 I.U. of enzyme.

Procedure: The final assay mixture contained 1 ml of potassium phosphate buffer (0.2 M, pH 8.2), 20 μl of tail homogenate (30 μl of tissues homogenate *viz.* liver, kidney and

intestine) and 50 µl of pyragallol. Distilled water, in equal volumes to the tissue homogenate, was taken for control experiment. The control was used to quantify the uninhibited auto-oxidation of pyragallol .This was used to determine the uninhibited auto oxidation of pyragallol. The reaction was started by the addition of pyragallol and the change in optical density was recorded for 180 seconds at 30 second interval. Change in the absorbance/minute was calculated from the reading. The SOD activity was expressed as IU per mg protein. One unit of SOD activity being defined as the amount of enzyme required to cause 50% inhibition of pyragallol auto-oxidation.

TOTAL SULFHYDRYL (-SH) GROUPS ESTIMATION

It was estimated according to the method described by Sedlak and Lindsey. (1968).

Principle: 5-5' dithiobis-2-nitrobenzoic acid (DTNB) is reduced by –SH groups of the samples to form 1 mole of 2-Nitro-5-mercaptobenzoic acid per mole of –SH. The reaction is carried out at pH 8.0. The nitromercaptobenzoic acid anion has intense yellow colour and can be used to measure –SH groups. The values are expressed as µg / mg protein.

Procedure: 0.5 ml of aliquots of tissue homogenate were mixed with 1.5ml of 0.2 M tris buffer having (pH 8.2) in 0.02M EDTA and 0.1 M DTNB (99 mg/25 ml methanol). The mixture was made up to 10.0 ml with 7.9 ml of absolute methanol. A reagent blank (without sample) and a sample blank (without DTNB), were prepared in a similar manner. The test tubes were allowed to stand with occasional shaking for 15 minutes. The reaction mixtures were centrifuged at approximately 3000 rpm, at room temperature for 15 minutes. Supernatant was collected and the absorbance of the supernatants was read in a spectrophotometer at 412 nm against blank (without DTNB) which was prepared in a similar manner. The test tubes were allowed to stand with occasional (intermittent) shaking for 15 minutes. Then the reaction mixtures were centrifuged again at approximately 3000 rpm at room temperature for 15 minutes. The absorbance of the supernatants was read in a spectrophotometer at 412 nm against blank (without sample).

REDUCED GLUTATHIONE (GSH) ESTIMATION [EC 1.13.11.18]

It was estimated according to the method Bentler and Gelbart, (1985).

Glutathione (γ-glutamylcysteinylglycine, GSH) is a highly concentrated intracellular antioxidant that accounts for 90% of intracellular non-protein thiol content. Highest concentration of GSH is present in liver. Glutathione exists in two forms: reduced

glutahthione (GSH) and the oxidized form-glutathione disulfide (GSSG). The GSSG/GSH ratio may be sensitive indicator of oxidative stress. Glutathione status is homeostatically controlled both inside and outside the cell, being continually self adjusting with respect to the balance between GSH synthesis (by GSH synthetase); its recycling from GSSG (by GSH reductase), and its utilization (by peroxidase, transferase, transhydrogenases and transpeptidases).

Principle: Glutathione is a major non-protein thiol present in the tissue. The sulphydryl group in glutathione reduces the 5,5,-dithio bis -2 nitro benzoic acid (Ellman's reagent, DTNB) to form one mole of 5-thio-2-nitro benzoate (TNB) per mole of SH by the reaction shown below:

$$2GSH + DTNB \longrightarrow GSSG + TNB$$

$$GSSG + NADPH + H^{+} \xrightarrow{GPX} 2GSH + NADP^{+}$$

Procedure: To 100 µl of whole blood / tissue supernatant in 900µl of D/W 1.5 ml of precipitating reagent was added and kept for 2 minutes. It was centrifuged at 3000 rpm for 10 minutes. The supernatant was recovered and to it 2 ml of phosphate solution was added. To 0.5 ml of this resulting supernatant, 250 µl of DTNB was added and the test tubes were read within 1 minute (of adding DTNB) at 412 nm in spectrophotometer. Absorbance was recorded against blank containing precipitating reagent, phosphate buffer and DTNB solution. The amount of enzyme activity was expressed in µg/mg protein.

GLUTATHIONE-S-TRANSFERASE (GST) ESTIMATION [EC 2.5.1.18]:

It was estimated according to the method Habig *et al.* (1974).

Principle: Glutathione transferase following its conjugation with the substrate 1, 2-dichloro-4-nitrobenzene shows an increased at absorbance at 340 nm.

Procedure: The assay mixture of 1 ml volume consists of 850 µl of 0.1 M sodium phosphate buffer containing EDTA, 50 µl of CDNB and 50 µl of tissue homogenate. The reaction mixture was maintained at 30°C and reaction was initiated by the addition of 50 µl GSH. The increase in absorbance was monitored for 3 minutes in a spectrophotometer. The enzyme activity was calculated using the extinction coefficient (E_{340} = 9.6 L $mmol^{-1}cm^{-1}$).

One unit of enzyme is defined as the amount of enzyme that catalyses the conjugation of 1 μM of CDNB per min. The enzyme activity was expressed as nM of CDNB-GSH conjugate formed/minute/mg protein.

GLUTATHIONE PEROXIDASE (GPx) ESTIMATION [EC 1.11. 1.9]

It was estimated according to the method Paglia and Valentine, (1967).

Principle: Glutathione peroxidase catalyses the reduction of various organic hydroperoxides, as well as hydrogen peroxides, as well as a hydrogen peroxide, with glutathione as hydrogen donor.

Procedure: The final assay mixture contained 100 μl of potassium phosphate buffer, 100 μl of GSH, 100 μl of sodium azide, 10 μl of tissue homogenate and 9 μl of distilled water. After adding all these above reagents the test tubes were incubated at 37° for 10 minutes and 100 μl of H_2O_2 was added. Tubes were incubated for 3 minutes at 37°C and 400 μl of precipitating reagent was added. The reaction was incubated for 5 minutes and than centrifuged. The supernatant were preceded as follows:

Supernatant	600 μl
0.4 M Sodium Phosphate	600 μl
DTNB	30 μl

The control tube had no enzyme

The blank tube had no GSH

The reactions were read at 412 nm. The enzyme activity was expressed as nmoles of GSH utilized/mg protein/ min.

PROTEIN ESTIMATION

It was estimated according to the method of Lowry *et al.* (1951).

Principle: It is the most commonly used technique to estimate the amount of protein present in the sample. The peptide bonds (-CO-NH-) present in the protein react with copper sulphate under alkaline condition to form a blue coloured complex. The Folin-Ciocalteau reagent which contains phosphomolybdate blue, and phosphomolybdic acid and phosphotungstic acid, reacts with the aromatic amino acid residues of the protein and gets reduced to phosphomolybdate blue and phosphotungstate blue products, which is quantified spectrophotometrically at 660 nm. The intensity of the colour is directly proportional to the amount of protein present in the sample.

Procedure: A set of tubes containing Bovine serum albumin (BSA) in the concentration range of (0-100 µg) was used. The volume in each tube was made to 1 ml with distilled water. 5 ml of freshly prepared alkaline copper sulphate solution was added in each of these tubes, mixed thoroughly and were incubated at room temperature for 10 minutes. In each of these tubes 0.5 ml of folin-ciocalteau reagent was added and the contents were mixed immediately. It was allowed to stand for 30 minutes at room temperature for the colour to develop. The absorbance of each tube at 660 nm was recorded. A standard curve of absorbance at 660 nm versus µg of BSA was plotted to determine the amount of protein in the sample.

SODIUM DODECYL SULFATE (SDS) POLY ACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Method: Laemmli, (1970).

Principle: This is a method used to separate proteins according to their size. Since different proteins with similar molecular weights may migrate differently due to their differences in secondary, tertiary or quaternary structure, SDS, an anionic detergent, is used in SDS-PAGE to reduce proteins to their primary (linearized) structure and coat them with uniform negative charges.

Procedures: SDS-PAGE generally involves 1) making a polyacrylamide gel and assembling the gel apparatus, 2) Boiling the protein samples in sample buffer containing SDS, glycerol and a reducing agent, 3) loading samples and running the electrophoresis, 4) fixing and staining the separated proteins. Commercially available protein molecular weight marker was obtained from Bangalore Genie.

PROTEIN CONTENT OF THE SAMPLE

Method: Bradford *et al.*, 1972.

1. **Bradford reagent:** Coomasie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml of 95 % ethanol. To the above sample 100 ml of 85 % (w/v) ortho phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre. (Final concentration: 0.01 % w/v coomassie brilliant blue G-250, 4.7 % (w/v) ethanol and 8.5 % (w/v) phosphoric acid.)

Estimation:

To a suitable amount of tissue supernatant (2-5 µl), 5 ml of Bradford reagent was added and mixed well. After 2-5 minutes, the absorbance was read at 595 nm. The standard curve was used to determine the protein in unknown samples (BSA as standard).

GELATIN ZYMOGRPAHY

Zymography is a simple, sensitive, quantifiable, and functional approach for the analysis of proteolytic activity in cell and tissue extracts, which was introduced more that 20 years ago. It is widely used to study extracellular matrix (ECM)-degrading enzymes, in particular the matrix metallo proteases (MMPs). MMPs are zinc-dependent endopeptidases capable of degrading ECMs, including the basement membrane. The MMP family consists of at least 26 members and has been classified into subgroups on the basis of substrate preference and molecular structure.

Procedure: The standard method for zymography is based on the use of SDS-PAGE gels co-polymerized with a protein substrate, in particular gelatin, casein, or fibrin. Proteases that have the ability to renature after removal of SDS and to exert proteolytic activity on a co-polymerized substrate can be analyzed with this method. MMP-2 (gelatinase A, 72 kD) and MMP-9 (gelatinase B, 92 kD) can be detected on gelatin zymograms and MMP-7 on casein gels. Coomassie Blue staining of the gel reveals sites of proteolysis as white bands on a dark blue background.

IMMUNOHISTOCHEMISTRY OF MMP-2 AND MMP-9

For all immunohistochemical and immunofluorencence procedure longitudinal cryostat sections were used. Briefly four to five sections 6-μm sections were cut from the tail stump and the sections were mounted on cleaned glass slides. After air drying for 15 minutes, the sections were fixed in ice-cold acetone for 10 minutes. The sections were air-dried again for 2-24 hours. Covered in aluminium foil the sections were stored at -20° C.

Staining for, MMP-2 and MMP-9 was performed using a standard Immunohistochemistry with AP-conjugated (NBT/BCIP) method (personal communication, Giorgio Cattoretti MD). In brief, the air dried sections were treated with 30% H_2O_2 containing 0.15 M sodium azide in methanol for 20 min to block endogenous peroxidase activity. For antigen retrieval, the slides were subjected to heat treatment in citric acid buffer, pH 6.0. After cooling down to room temperature, the slides were incubated for 7 min with an undiluted blocking solution containing bovine serum albumin. After rinsing with phosphate-buffered saline (PBS), the slides were incubated with a primary antibody against MMP-2 (A generous gift from Dr

Sibsankar Roy; IICB, Kolkata, 1:100 dilution of rabbit anti-MMP-2, Sigma) or MMP-9 (A generous gift from Dr. Snehasikta Swarnakar, IICB, Kolkata; 1:100 dilution of Polyclonal goat Antihuman anti-MMP-9 Ab, Santa Cruz.). After incubation with the primary antibody, slides were rinsed twice with PBS and incubated overnight in a humid chamber. Without letting the sections dry out, the ALP conjugated secondary antibodies (1:500) was added and the slides were incubated for at least 45 minutes and the localization were visualized using 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium substrate solution from Sigma (incubation for 10-20 minutes until desired positive staining was attained).The sections were then visualized in Leica DMRB, microscope and photographed using a CCD camera.

IMMUNOFLUORENCE OF DESMIN and MYOSIN HEAVY CHAIN

Following an initial tissue processing (as in case of MMP-2 and MMP-9) the sections were incubated with primary antibody against desmin (A generous gift from Dr. Jyotsna Dhawan, CCMB, Hyderabad; 1:300 dilution of Anti Desmin) or against myosin heavy chain (A generous gift from Dr. Jyotsna Dhawan, 1:300 dilution of Anti Myosin) for 18 hours overnight inside a moist chamber. The moist sections were subsequently incubated with fluorescent conjugated secondary antibodies (goat anti-rabbit Alexa Fluor 488 , 1:500 dilution).The sections were wrapped in aluminium foil to prevent exposure to light and later observed under a fluorescent microscope (Leica, DMRB) using a filter of 515–560 nm wavelength of light .

IMMUNOFLUORENCE OF VEGF

As in case of Desmin and Myosin the sections were incubated with primary antibody (A generous gift from Dr. Gopal Kundu, NCCS Pune; 1: 50 dilution of Anti VEGF, Oncogene) for 16 hours overnight inside a moist chamber. Following overnight incubation the sections were incubated with FITC-conjugated secondary antibody (1:100). The sections were wrapped in aluminium foil to prevent light exposure and later observed under a fluorescent microscope (Leica, DMRB) of 515–560 nm wavelength of light.

HISTOLOGY

Decalcification of the regenerate along with the fixation of the tail tissue was performed. The formalin fixed tissues were washed and subjected to dehydration in ascending grades of alcohol. The tissues were blocked in paraffin wax, sectioned into ribbon and fixed on glass

slides. After staining and destaining with hematoxylin and eosin, they were permanently mounted with DPX mount for histopathological studies.

Procedure: Approximately, 5 to 10 mm^3 of tissue, fixed in 10 % formalin was washed thoroughly in running water. For tail sections proper decalcification was followed by using strong acid in 10% constitution. The fixed tissue samples were dehydrated by placing them in increasing concentrations of alcohol (30, 50, 70, 90 and 100 %) for clearing and subjected to cold infiltration at room temperature in a mixture containing paraffin wax dissolved in Xylene for 30 minutes. The tissues were hot infiltrated in oven at 58°C to 60°C in molten paraffin wax for 1 hour. The hot infiltrated tissues were quickly removed and blocked quickly using metal blocks. The solidified blocks were trimmed to 8 to 10 micron thickness. The ribboned sections were placed on glass microscopic slides coated with egg albumin.

The microscopic slides were then exposed to decreasing order of alcohol (100, 90, 70, 50 and 30%) for 5 minutes. The sections were dipped in Hematoxylin stain for 5 min and washed again in running tap water to remove the excess stain. The slides were dried and dehydrated in increasing concentration of alcohol (30, 70, and 90 for few seconds). After drying, the slides were cleared in Xylene twice for 10 minutes. These cleared tissue sections were permanently mounted with DPX. The permanently mounted sections of the tissues were observed under light microscope for histological evaluations.

IMMUNOHISTOFLUORESCENCE STUDIES

FLUORESCENCE MICROSCOPY ANALYSIS OF NUCLEAR ALTERATIONS DURING CELL PROLIFERATION:

Acridine Orange (AO) Staining:

Principle: Acridine Orange (AO) is a nucleic acid selective metachromatic stain useful for cell cycle determination. AO interacts with DNA and RNA by intercalation and electrostatic attraction respectively. DNA intercalated AO fluoresces green (525nm); RNA electrostatically bound AO fluoresces red (>630nm). AO may be used to distinguish between

quiescent and activated, proliferating cells, and may also allow differential detection of multiple G_1 compartments.

Procedure: The tail regenerate was removed immediately after sacrificing the animals, blotted dry, and transferred to a cryostat microtome maintained at -20^0 C. The tissues were embedded in OCT compound (Tissue Tek-II) and sectioned at 16μm in longitudinal plane. The sections were fixed in Carnoy's fixative and passed on to different grades of alcohol from 90% to 30% (time) and kept for 2 minutes in buffer I and for 10mins in buffer II and for 15 minutes exposed to 0.1% Acridine Orange in 0.1M phosphate buffer (pH-6.0). The sections were than observed under fluorescence microscope (Leica, DMRB). DNA of cells was identified as brilliant green and cellular RNA as brilliant orange.

***In vivo* DNA replication labelling**

***In vivo* BrdU labelling:**

Method: Santamaria *et al.*, 1996.

Principle: Bromodeoxyuridine (BrdU) labeling method is based on the principle of nucleotide substitution of thymidine with uridine in the DNA structure of dividing cells both *in vitro* and *in vivo.* BrdU has been utilised in a number of *in vitro* and *in vivo* studies to label a variety of cellular sources from human olfactory epithelium and neural progenitors to neural stem cells.

Procedure: BrdU (Sigma, St Louis, USA) was dissolved in Phosphate buffered saline (PBS) and was injected intra peritoneal, at a dose of 100 mg / Kg body weight at different stages of the regeneration. The regenerating tail was dissected and the zone of interest was fixed in 10% formalin in PBS. Fresh frozen cryostat sections (10μm thick) were mounted on 2% silane coated slides. The sections were blocked in PBS for 30 min at RT. The sections were than incubated with BrdU MAb (mouse monoclonal antibody) (1:500) overnight at 4^0 C. The sections were than washed in PBS for 20 minutes. Washing was followed by incubation in FITC labeled secondary antibody (Ab). The sections were washed again in PBS for 5 min. The nuclei were stained with Hoechst nuclear stain for 20 min. After three rinses in PBS, the sections were mounted using a solution of 50 % Glycerol/50% PBS and immediately photographed under a fluorescent light on a Leica DMRB microscope.

STATISTICAL ANALYSIS

The significance level of the experiment and control group was evaluated by Student's 't' test, with a 95% confidence limit. For multiple group comparison and difference between the groups the data were subjected to One Way Analysis (ANOVA) followed by Duncan's Multiple Range Test (Duncan, 1955) using SPSS-PC Statistical Analysis Package. A 'p' value of 0.05 or less was accepted as being statistically significant. Graphs are prepared by using Origin 7.0 SRO origin Lab Corporation One Round House Plaza, Northampton MA USA.