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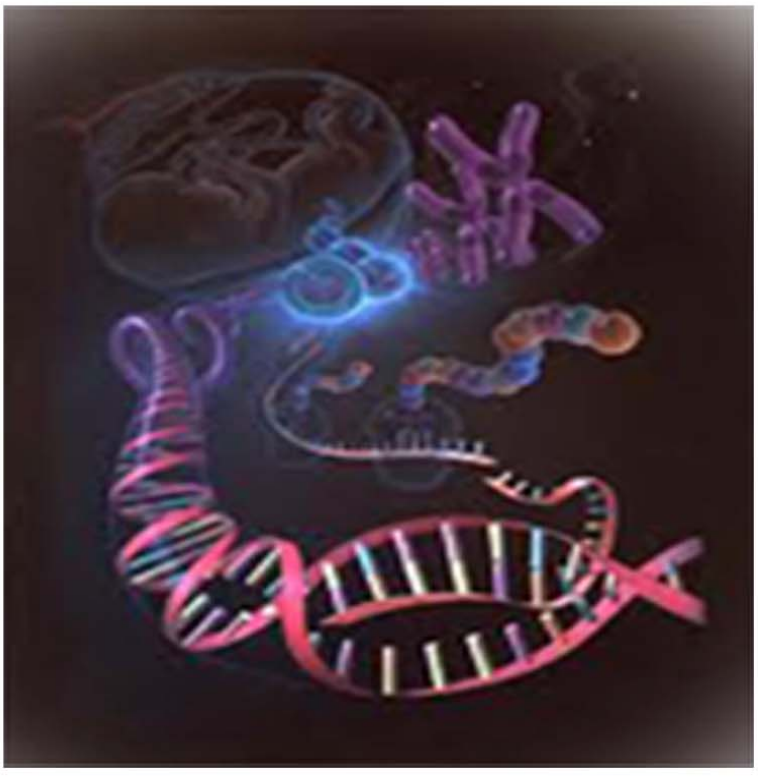

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Research Paper

PHARMACOLOGICAL INHIBITION OF FGFR1 SIGNALING ATTENUATES THE PROGRESSION OF TAIL REGENERATION IN THE NORTHERN HOUSE GECKO *HEMIDACTYLUS FLAVIVIRIDIS*

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Fibroblast Growth Factor 2 (FGF2) is one of the key modulators of epimorphic regeneration. The current study was focused on investigating the role of FGF2 signaling in reptilian regeneration, a poorly explored amniote model system to study epimorphosis. FGF2 signaling pathway was targeted via inhibition of FGF receptor 1 (FGFR1) using the pharmacological inhibitor SU5402. Morphometric studies revealed that FGF2 is important for wound healing and initial proliferative activities leading to differentiation, as both these processes were found hampered in SU5402 treated animals. However, the late differentiation was found independent of FGF2 signaling. Further, a careful observation on the histological profile of the regenerates was done to understand the effect of impaired FGF2 signaling on the tissue differentiation and restoration. It was apparent from the study that the ablation of FGF2 signal hampers important processes of epimorphosis like formation of a wound epithelium, recruitment of blastemal cells and their further proliferation and differentiation, all of which eventually lead to restoration of the lost appendage. Conclusively therefore, FGF2 seems to be a necessary molecule for successful reptilian regeneration and could be an essential requirement for appendage regeneration across varied classes of vertebrates which exhibit epimorphosis.

Keywords: SU5402 Epimorphic regeneration, FGF2, *Hemidactylus flaviviridis*

INTRODUCTION

For centuries, the phenomenon of epimorphic regeneration, a complete reformation of lost tissues and organs after the development of body plans and cellular differentiation, has been a mystery of life and has fascinated many biologists (Nakateni *et al*., 2007). In invertebrate species including the hydra, planarians and arthropods, the regeneration of lost tissues and parts is widely observed (Slack, 2003), whereas most vertebrate species do not have such a remarkable ability for regeneration. Mammals only retain limited regeneration ability in the adult liver and infant fingertips (Hata *et al*., 2007; Yoshizato, 2007).

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However, among vertebrates, urodeles, lizards and fish have high regeneration abilities and express epimorphic regeneration. Epimorphic regeneration involves generation of new stem cells, either by proliferation of the existing stem cells or by dedifferentiation of adult cells, which differentiate to form the lost appendage that is more or less similar in size and structure compared to the original lost structure (Brockes and Kumar, 2002; Bryant *et al*., 2002).

A review of literature is clearly indicative of the interest to understand the regenerative ability of amphibians and the processes involved therein. However, such studies for reptiles have not been extensive, despite the fact that tail autotomy and regeneration among many lizards is a widely known phenomenon and lizards represent an excellent non mammalian amniote model to study epimorphosis. Moreover, the process of regeneration is comparable between the lizards and amphibians (Iten and Bryant, 1976). Defined stages seen during lizard tail regeneration include (a) Wound Epithelium (WE): during which wound closure, inflammation, dedifferentiation and blastemal cell accumulation occur; (b) Blastema (BL): during which proliferation of blastemal cells leads to elongation of the blastema; (c) Differentiation: a morphogenetic phase leading to regenerative outgrowth and repatterning (Sharma and Suresh, 2008). The precise coordination of several events involved in such a complex process requires cross talk and signaling between many factors and differential regulation of several genes.

Neurotrophic factors derived from the nerve tissue are one such regulatory factors of regeneration. Some earlier studies using amphibian model prompted many to believe that the main neurotrophic factors responsible for the orchestration of regeneration could be Fibroblast Growth Factors (FGFs), especially the prototypic FGFs, FGF1 and FGF2 (Brookes, 1984; Geraudie and Ferretti, 1998; Mescher, 1996). FGF2 is known to play key roles in development, remodeling and disease states in almost every organ system. It is a potent angiogenic molecule *in vivo* and *in vitro,* stimulates smooth muscle cell growth, wound healing, and tissue repair (Basilico and Moscatelli, 1992; Schwartz *et al*., 1993). The biological activity of FGF2 requires the presence of both Heparan Sulfate Proteoglycans (HSPGs) and FGF tyrosine kinase receptors (FGFRs) to transduce signals for cell proliferation (Guillonneau *et al*., 1996; Ornitz and Itoh, 2001; Ornitz *et al*., 1992). FGFR1 and FGFR2 bind FGF2 with the greatest affinity, but the level of redundancy in receptor utilization within the FGF family is high (Ornitz *et al*., 1992). An increasing number of studies have targeted the FGF2 pathway through inhibition of the tyrosine kinase activity of FGFR1 (Izikki *et al*., 2009; Mohammadi *et al*., 1997; Mori *et al*., 2007; Woad *et al*., 2009).

Besides its many roles in several physiological and developmental processes, FGF2 is also one of the key players of epimorphic regeneration. FGF2 has been localized to the WE and nerves of the regenerating amphibian limb and it can re-establish the expression of several genes, which had been inactivated after denervation, thus allowing denervated limbs to regenerate (Mullen *et al*., 1996). FGF2, in addition to being up-regulated in the regenerating spinal cord in newts, is also expressed in a subset of blastemal cells and chondroblasts, in the basal epidermal layer and also in differentiating muscle (Ferretti *et al*., 2001). FGF2 soaked beads can stimulate chick limbs, which normally do not regenerate, to do

so (Kostakopoulou *et al*., 1996; Taylor *et al*., 1994). Implantation of FGF2 soaked beads can even induce extra limbs from the flank of chick embryo *in vivo* (Cohn *et al*., 1995). Furthermore, FGF2 is known to promote blastemal growth during zebrafish fin regeneration as well (Hata *et al*., 1998).

Hence, in light of the role of FGF2 in epimorphosis of diverse animal models, the current study was aimed to unearth its possible role in reptilian tail regeneration using northern house gecko, *Hemidactylus flaviviridis,* as the animal model. FGF2-FGFR1 signaling pathway was targeted using specific tyrosine kinase inhibitor SU5402 and the effects on successive stages of regeneration were observed. Further, it is also important to evaluate the histological details underlying the regenerative process. Histological descriptions of the regenerative response have been previously illustrated for several lizard species (Alibardi and Toni, 2005; Bellairs and Bryant, 1985). However, such details for *H. flaviviridis* tail regenerate involving FGF2 inhibition have not been worked out and it becomes important to explore the structural alterations occurring in the tail regenerate due to the administration of FGFR1 inhibitor.

Thus, the current study is an attempt to establish the significance of FGF2 in tail regeneration of a reptilian model *H. flaviviridis* that will also help one understand whether FGF2 signaling is quintessential for epimorphic regeneration in vertebrates that exhibit regenerative ability. Further, lizard tail is projected as a potential model to study regeneration with a view to develop possible treatments for human diseases (Daniels *et al*., 2003). Moreover, understanding the regeneration mechanisms of lower vertebrate model systems is always aimed at achieving basic cues that could be useful in regenerative medicine.

MATERIAL AND METHODS

Experimental Animals

Adult northern house gecko *Hemidactylus flaviviridis* Rüppel, of both the sexes, with normal intact tail, weighing 10 ± 2 g were procured from local animal dealer. All animals were screened for parasitic infestation and the healthy ones were acclimated for a week before the commencement of the experiments. The animals were housed in well ventilated wooden cages with glass slider on one side for light and visibility. The cages were maintained in the Departmental animal house at controlled temperature (30 ± 2^{o}C) and light–dark schedule (12:12). These environmental conditions evoked optimal regeneration in lizards. They were fed with cockroach nymphs twice a week and purified water was given daily *ad libitum.* The experimental protocols used in the current study were carried out in accordance to the ethical guideline of Drugs and Cosmetics Rules, 1945 and, was reviewed and approved by the Institutional Animal Ethics Committee (No. ZL/IAEC/14-2010).

Treatment Before Amputation

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

Group 1: This group of animals served as a control to the experimental group and was injected with vehicle (2% DMSO).

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

A stock solution of SU5402 (Calbiochem®, EMD Biosciences, Inc., US) was prepared in DMSO and stored at 4°C. This stock was diluted to obtain a final dilution of SU5402 in 2% DMSO, which was used for treatment.

Autotomy was induced in control and experimental animals by pinching the normal intact tail, two segments away from the vent. The drug dosage was selected based on an initial dose range study. Drugs were administered *in loco* (at second intact tail segment from vent) at a maximum quantity of 0.075 ml/animal. Treatment started two days prior to amputation and was continued on every alternate day till the termination of the experiment. The growth of the regenerate was measured at fixed intervals using a calibrated digital Caliper (Mitutoyo, Kawasaki, Japan) and time taken to reach defined stages of regeneration was recorded.

Stage Specific Treatment

Autotomy was induced, as described earlier, in 50 lizards, and the regenerating animals were selected at two defined stages of regeneration *viz*., (i) at completion of wound healing and appearance of WE stage (WE appears as a smooth shining surface), and (ii) at early blastema (BL) stage (regenerate of about 2-3 mm length) . Only those animals that attained the above stages on the same day were selected and grouped.

Treatment at WE Stage

Treatment commenced at WE stage and was continued on every alternate day till the animals were sacrificed. Eighteen animals that reached the WE stage on the same day were selected and divided into two groups of nine animals each and were treated as in 4.2.1.

Treatment at BL Stage

Eighteen lizards that attained the blastema stage on the same day were selected for the experiment. They were divided into two groups of nine animals each and treated as in 4.2.1. Treatment started at the blastema stage and continued on alternate days as described earlier.

The time taken to reach the various stages of tail regeneration and the rate of growth of regenerate were recorded at fixed intervals.

Histological study of tail regenerate of Control and SU5402 treated *H. flaviviridis*

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

Group I: This group of animals served as control to the experimental groups and injected with vehicle (2%DMSO).

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

The procedures for autotomy and treatment remained same as stated earlier. Control animals reaching the different stages of regeneration were selected and sacrificed as per standard protocol (Reilly, 2001). The treated animals were sacrificed on the day when the control animals reached the appropriate stages irrespective of whether treated animals attained the same stage or not. The regenerate was processed for histological analysis. Extra animals were euthanized and incinerated.

Samples were fixed and decalcified. Paraffin wax blocks of the tissue samples were prepared. Longitudinal sections (7 µm) of tails from three animals per regeneration group were stained with Harris's haematoxylin and eosin. The histological structure of the tissues on the slide was

visualized using Leica DM2500 Microscope and pictures captured using EC3 Camera (utilizing LAS EZ software).

STATISTICAL ANALYSIS

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

RESULTS

Progression of Regeneration in *H. flaviviridis* Subjected to Treatment Before Amputation

In this experiment, SU5402 treatment started prior to amputation and it was observed that blocking FGF-2 signaling significantly hampered the progression of epimorphosis. Rate of growth and percentage of growth inhibition were calculated for the growth (2-12 mm) and differentiation (12-24 mm) stages of regenerating tail. It was observed that SU5402 treatment significantly decreased the growth rate of regenerate during both 2-12 mm ($p<0.01$) and 12-24 mm stages of growth ($p<0.05$). A significant decrease was observed in the length of the regenerate of all SU5402 treated animals with 59% reduction in growth rate during 2-12 mm stage and 27% reduction during 12-24 mm stage (Tables 1 and 2).

Progression of Regeneration in *H. flaviviridis* Subjected to Stage-specific Treatment

To understand the role played by FGF-2 at different stages of tail regeneration, lizards were treated at distinct stages of regeneration, viz., WE and BL.

Treatment at WE Stage

Injection with FGF-2 receptor inhibitor at the

Table 1: Number of Days Taken to Reach Various Regenerative Stages in *H. flaviviridis*, Subjected to *in loco* Injection of SU5402 Before Amputation

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

Table 2: Length of Tail Regenerated in *H. flaviviridis* After *in loco* Treatment with SU5402 Before Amputation

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

Note: [a] Wound healing; [b] Values are expressed as mode and range in parenthesis; [c] Values are expressed as Mean ± SE, n=5, * p ≥ 0.05, **p ≥ 0.01; [d] Values are corrected to the nearest whole number.

wound epithelium stage delayed attainment of subsequent stages of regeneration. Lizards treated with SU5402 took an additional 5 days to attain the blastema stage as compared to control animals. Moreover, the treatment group reached the differentiation stage on an average of 23 days, whereas control animals took only 13 days to attain the same. Further, a definite decrease ($p<0.01$) in the rate of growth of regenerate was observed in SU5402 treated animals with a mean reduction of 46% during 2-12 mm stage and 17% during 12-24 mm stage of regeneration (Tables 3 and 4).

Treatment at BL Stage

In contrast to the first two experiments, treatment with SU5402 at the blastema stage showed only marginal influence on the progression of regeneration. A delay of two days in attaining the differentiation stage was observed in the treatment group. However, even though the progression of growth was hampered during initial stages of growth, no statistically significant reduction in rate of growth was recorded during 12-24 mm stage (Tables 5 and 6).

Histological Observations in Tail of Control and SU5402 Treated *H. flaviviridis*

At Wound Epithelium (WE) Stage

Control tail sections at this stage showed defined structures like a well formed WE, which was a few layers thick at the apex, aptly called the Apical Epithelial Cap (AEC). The regenerating ependymal tube, several blood vessels and connective tissue were also evident. Further, cells accumulating towards the wound epithelium could also be seen, which will eventually form the blastemal cone. However, the wound surface of tail sections from treated animals showed traces of blood clots indicating that the wound is yet to heal. No distinct ependymal tube was present in the tail sections of treated lizards. Blood vessels nourishing the regenerate were also found significantly reduced. Ultimately all these affected structures are important determinants necessary

Table 3: Number of Days Taken to Reach Various Regenerative Stages in *H. flaviviridis*, Subjected to *in loco* Injection of SU5402 at WE Stage

Treatment	No. of Days		
	WH[a]	BL (2mm)	DF (12mm)
Control	5(5-6)[b]	9 (8-9)	13 (13-14)
SU5402 Treated	5(5-6)	14(14-15)	23 (22-23)

Table 4: Length of Tail Regenerated in *H. flaviviridis* After *in loco* Treatment with SU5402 at WE Stage

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

Note: [a] Wound healing; [b] Values are expressed as mode and range in parenthesis; [c] Values are expressed as Mean±SE, n=5, * $p \leq 0.05$, ** $p \leq 0.01$; [d] Values are corrected to the nearest whole number.

Table 5: Number of Days Taken to Reach Various Regenerative Stages in *H. flaviviridis*, Subjected to *in loco* Injection of SU5402 at BL Stage

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

Table 6: Length of Tail Regenerated in *H. flaviviridis* After *in loco* Treatment with SU5402 at BL Stage

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

Note: [a] Wound healing; [b] Values are expressed as mode and range in parenthesis; [c] Values are expressed as Mean±SE, n=5, * $p \leq 0.05$, ** $p \leq 0.01$; [d] Values are corrected to the nearest whole number.

Figure 1: Various stages of tail regeneration; Vehicle Control (DMSO treated) *H. flaviviridis*. Gross morphology of the regenerating tail in dorsal view (A-C) and histological details of the same (D-F); (A) Wound epithelium seen as a shiny smooth surface (5days post amputation (dpa)); (B) Blastemal cone 3-4mm in length (8dpa); (C) Differentiation stage with a 12-13mm long regenerate (13dpa); (D) A proper aec is prominent after wound healing; (E) Proliferating blastemal cells with regenerating muscles, ependyma and procartilage are evident during blastema; (F) Proper regenerating nervous, muscle and connective tissue along with a well formed epithelium are seen during differentiation. aec- apical epithelial cap; at- adipose tissue; ba- cell accumulation prior to blastema formation; bl- blastema; c- cartilage; ct; connective tissue; e- ependyma; ep- epidermis; de- dermis; mb- muscle bundle; nt- neural tube; pc- procartilage; pm- promuscle; sg- spinal ganglion; rc- regenerating connective tissue; we- wound epithelium. Arrows indicate blood vessels. Scale bars: d-f = 1mm

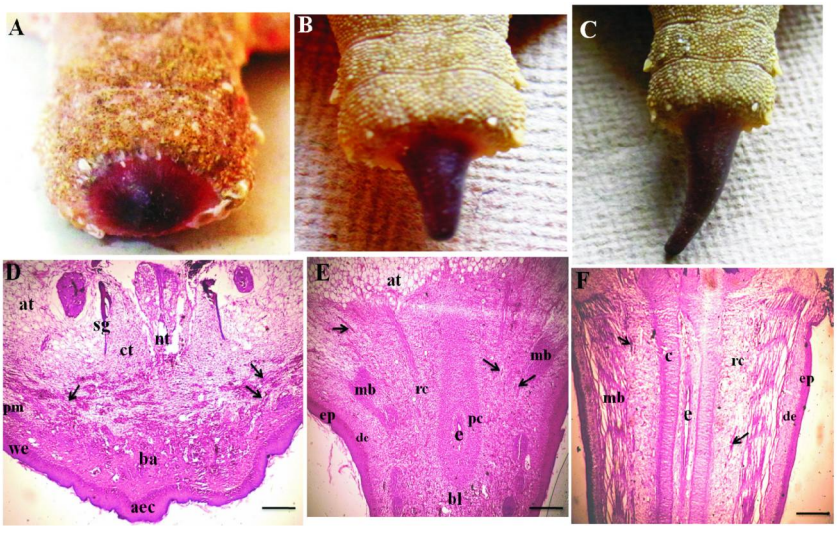
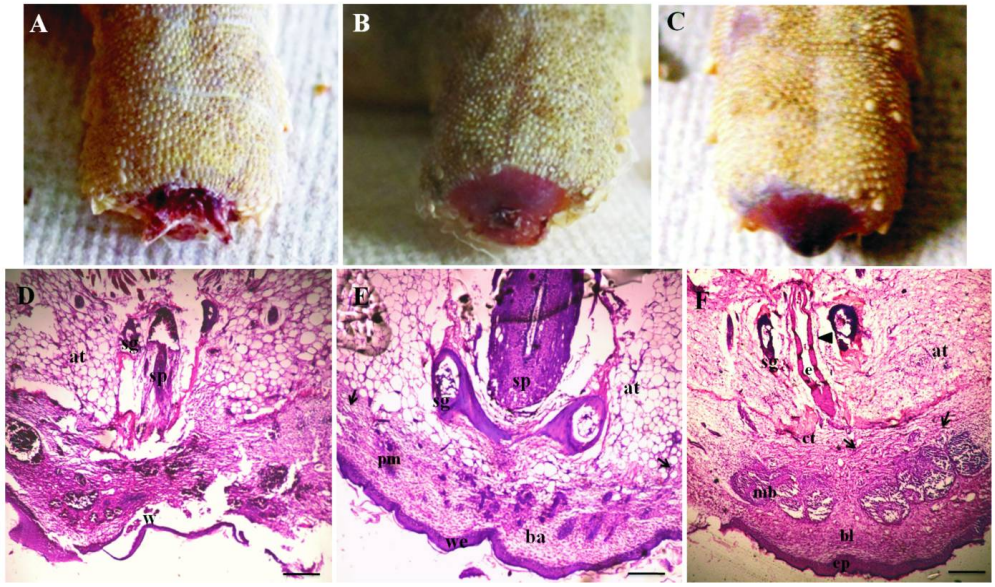


Figure 2: Various stages of tail regeneration. SU5402 treated *H. flaviviridis*; Gross morphology of the regenerating tail in dorsal view (A-C) and histological details of the same (D-F); (A) Wound not healed in SU5402 treated animal (5days post amputation (dpa)); (B) A proper wound epithelium still not evident (9 dpa); (C) Blastema formation and proliferation is delayed in SU5402 treated animals with blastema evident only after 15dpa; (D) Wound not healed with traces of blood clot seen; (E) A thin WE seen along with accumulation of cells to form the blastema; (F) Improved stage of regeneration seen with an epithelium and regenerating ependyma, muscle bundles and traces of cartilage tissue (arrowhead). at- adipose tissue; ba- cell accumulation prior to blastema formation; bl- blastema; ct; connective tissue; e- ependyma; ep- epidermis; mb- muscle bundle; pm- promuscle; sg- spinal ganglion; sp- spinal cord; w- wound yet to heal; we- wound epithelium; Arrows and indicate blood vessels; Scale bars: d-f = 1mm



for eventual proliferation. Thus, accumulation of cells leading to blastema too was not prominently seen in the treated caudal sections (Figure 1d, 2d).

At Blastema Stage

Control sections at blastema stage showed a thicker, multilayered WE and a thin layer of dermis beneath. The ependymal tube was properly evident. An additional feature noted during this stage was the appearance of procartilagenous tissue accrued surrounding the ependyma. Regenerating muscle bundles, seen as separate aggregates beneath the dermis, are another prominent histological feature seen during this stage. Several blood vessels and a pool of proliferating blastemal cells immediately beneath the WE could also be seen. In the treatment group, by this time, initial signs of wound healing begin to appear. A thin WE was formed, with few blood vessels seen in the region beneath it. A small area of proliferating cells immediately following a faintly regenerated ependymal tube was also evident. However, cartilage and muscle tissue were still not manifested (Figure 1e, 2e).

At Differentiation Stage

This stage accounts for the complete re-growth of the lost tail with proper tissue architecture. A

well developed epithelium including both the epidermis and dermis was observed. Pronounced ependymal growth could be seen with well developed cartilaginous tissue surrounding it. Properly regenerated muscle bundles with well formed connective tissue and adipose tissue were observed. With the formation of muscle bundles, the regenerate from the treatment group at this stage showed signs of mesenchyme differentiation. An elongating ependyma finally becomes evident with traces of regenerating cartilage surrounding it. The zone of proliferating blastemal cells was also seen evidently beneath a proper epithelium (Figure 1f, 2f). However, the tissue architecture observed in the SU5402 treated tail at differentiation stage is poorly organized compared to that of control at the same stage and is equivalent only to a tail at the blastema stage, indicating a definite delay in regeneration. A dorsal view of the gross morphology of the regenerating tail during different stages has also been shown in Figures 1a-c and 2a-c indicating the delayed regeneration in treated animals.

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

DISCUSSION

Involvement of FGF2 in several developmental as well as regenerative processes such as limb development, angiogenesis, wound healing, and repair is well established (Obara *et al*., 2003; Yokoyama, 2008). That FGF2 is one of the main neurotrophic factors affecting epimorphic regeneration in amphibian and fish model systems is also a well known fact (Hata *et al*., 1998; Mullen *et al*., 1996). The present study examines whether this candidate neurotrophic factor of epimorphosis, functions in a similar manner during tail regeneration of *H. flaviviridis*, a reptilian model of regeneration which unlike fish and amphibian is an amniote that is evolutionarily higher in hierarchy and hence, might have evolved a different regulatory mechanism for epimorphosis.

Morphometric observations amply testify that FGF2 signaling is imperative for regenerative response in *H. flaviviridis*. Inhibition of FGF2 signal by treatment with SU5402 hampered the wound healing process and delayed the formation of a proper wound epithelium during tail regeneration. Formation of a functional wound epithelium is essential for successful regeneration as it provides the necessary signals for the underneath tissues to dedifferentiate, proliferate and to form the blastema (Kumar *et al*., 2000; Lo *et al*., 1993). Impaired wound healing in the SU5402 treated lizards must be due to inadequate FGF2 signaling. FGF2 is a potent

mitogen and is involved in epithelial cell proliferation and migration taking place during wound healing. It has also been shown that FGF2 incorporated chitosan hydrogel may be a promising wound dressing, especially in the treatment of healing impaired wounds (Obara *et al*., 2003). Besides, the process of wound healing is known to be controlled by critical events like re-epithelization, angiogenesis and matrix deposition (Miller and Gay, 1992), and it has been hypothesized that FGF-2 might be involved in these processes.

Moreover, wound healing is closely complemented by cell accumulation, leading to the appearance of the blastemal cone. SU5402 treatment both before amputation and at WE stage delayed this initial growth and blastema formation. Blastema formation is a major cue in epimorphic regeneration. It has been postulated that the basis for regenerative competence resides in the capability of an adult organism to induce a local and discrete population to re-enter the cell cycle in a highly controlled manner (Brockes, 1997; Stocum, 1999; Tsonis, 1996). The proliferative blastemal cells can then eventually give rise to all of the cell types necessary for the complete regeneration of the lost structure (Clause and Capaldi, 2006). During newt limb regeneration, the FGF2 receptor, FGFR1, is expressed in blastema cells, suggesting that it could be acting on blastemal tissues to promote mitotic activity (Poulin *et al*., 1993). FGF2 is also reported to be the endogenous mitogenic factor responsible for blastema formation and growth in amputated and denervated early limbs of *Xenopus laevis* (Cannata *et al*., 2001). It regulates blastemal proliferation during fin regeneration as well (Poss *et al*., 2000; Tawk *et al*., 2002). In the current study also, FGF2 signal inhibition delayed attainment of blastemal stage in *H. flaviviridis*. Evidently, the pool of accumulating blastemal cells and their proliferation leading to formation of blastemal cone was affected by impaired FGF2 signaling. Further, the formation of blastema involves extensive remodeling of the extracellular matrix, during which activity of Matrix Metalloproteinases (MMPs) is required. Since, FGF2 is known to increase the activity of MMPs (Palmon *et al*., 2000), it is possible that the process of matrix reorganization in the animals treated with SU5402 might have been affected which could be an additional reason for delayed the formation of blastema in treated lizards.

Further, once the blastema is formed, the cells get engaged in repeated cycles of cell division leading to the increase in the length of the regenerate. Injection of SU5402 before autotomy and at WE stage, curtailed the rate of growth of regenerate. This decrease in the growth rate of regenerate was more significant from 2-12 mm. Growth rate of regenerate from 12-24 mm was also decreased in the treated lizards, but it was not significant statistically. This prompted one to propose that once the regenerate accomplish a certain length and commence differentiation it no more requires FGF2 signaling for the furtherance of its growth. This notion gains credence from the current stage specific treatment wherein it was observed that the growth rate of regenerate in animals that received SU5402 at blastema stage was not significantly different from that of the control animals. Conversely, Kruzhkova and Burgess (2000) from their study concluded that FGF2 inhibited the process of skeletal muscle differentiation in chick. Recently it has been

shown that HSPG glypican-1 acts as a positive regulator of muscle differentiation by sequestering FGF2 in lipid rafts and preventing its binding and the dependent signaling (Gutierrez and Brandan, 2010). Moreover, terminal differentiation of MM14 mouse myoblasts is repressed by pure preparation of FGF2 (Clegg *et al*., 1987).

In order to complement the morphometric observations and to identify the target tissues affected by impaired FGF2 signaling, histological profile of the tail sections was also analyzed in the present study. The observations revealed that the several events of epimorphosis like formation of the wound epithelium, recruitment and proliferation of blastemal cells, differentiation of mesenchymal cells as muscle bundles, ependymal growth and regeneration of the supporting cartilage surrounding the ependyma are delayed in the SU5402 treated lizards. Blood vessel formation is also evidently being downplayed in the treatment group. These results are indicative of the importance of FGF2 signaling in the timely restoration of a lost appendage with proper tissue integrity even in a reptilian model.

A careful scan through the archived reports revealed that FGF2 indeed play a definite role in many of the developmental events reported in the current study. Alibardi (1999 and 2001) while studying limb regeneration in newts noted that the wound epidermis start as an immature keratin layer and the formation of the granulated layer occurs by the accumulation of keratohyalin-like granules. FGF2 has been reported to induce differentiation of keratinocytes (Werner *et al*., 1993). Moreover, FGF2 has been localized to the apical cup during new limb regeneration and is reported stimulating blastemal cells to respond to cell replication factors (Giampoli *et al*., 2003).

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

There are many reports regarding the involvement of FGF2 in the regeneration of nerves during epimorphosis. It plays a role both in the early stages of regeneration, possibly in the proliferation of neural progenitors and in the maintenance of the undifferentiated state (Ferretti *et al*., 2001). Zhang *et al*. (2000) observed induction of FGF2 expression in the regenerating spinal cord and in the undifferentiated cells lining the ependymal canal of amphibian tail regenerate. FGF2 in addition to being up-regulated in the regenerating spinal cord is also found expressed in a subset of blastemal cells and chondroblasts, in the basal epidermal layer and even in differentiating muscles in amphibians (Ferretti *et al*., 2001). It is also been reported that FGF2 is a potent regulator of diverse functions of bone and cartilage cells. FGF2 is produced by cells of osteoblastic lineage, accumulated in bone matrix and acts as an autocrine/paracrine factor for bone cells (Canalis *et al*., 1988; Hurley *et al*., 1994; Rodan *et al*., 1989).

CONCLUSION

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

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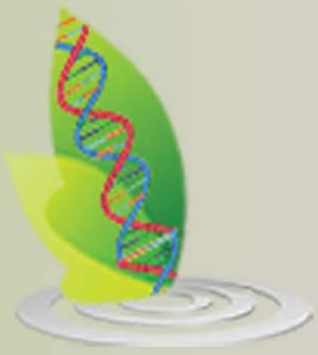

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