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Studies on tail regeneration and homeotic transformation in anuran tadpoles

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ABSTRACT Anuran tadpoles are excellent models for regeneration studies. The tail, an organ essential for swimming for the aquatic tadpole, regenerates completely following injury or amputation. However, treatment with the morphogen, vitamin A or retinoic acid inhibits normal tail regeneration and induces homeotic transformation of tail to limbs. This phenomenon was discovered for the first time in the Indian marbled balloon frog *Uperodon systoma* in the Developmental Biology laboratory of Utkal University (Odisha, India) in the year 1992. In this paper, we present the results of morphological, histological, biochemical and molecular (immonohistochemistry) investigations of vitamin A induced homeotic transformation in different anuran species. In addition, we discuss the putative role of fibroblast growth factor 1 during spinal cord regeneration in the tadpoles of the Indian tree frog, *Polypedates maculatus*, an ideal model for regeneration studies in an Indian context.

KEY WORDS: *anuran tadpole, tail regeneration, homeotic transformation, vitamin A/retinoic acid*

Introduction

Vitamin A induced homeotic transformation of tail to limbs in the marbled balloon frog *Uperodon systoma* was observed for the first time in our laboratory (Mohanty-Hejmadi *et al.*1992). Following the initial finding, there were several reports on vitamin A induced inhibition of tail regeneration and homeotic transformation in different anuran species namely *Polypedates maculatus, Bufo melanostictus* (present name *Duttaphrynus melanostictus*), *Microhyla ornata* (Mahapatra, 1994; Mahapatra and Mohanty-Hejmadi, 1994, Mahapatra *et al.,* 2001; Mohanty-Hejmadi and Crawford, 2003) and *Rana tigerina* (present name *Hoplobatrachus tigerinus*) (Das and Dutta, 1996). We investigated morphological, histological, biochemical and molecular aspects of tail regeneration in the anuran tadpoles.

Collection of egg nests and rearing of tadpoles

Egg nests of different anuran species were collected during the rainy season i.e., months of July to September from different breeding grounds inside the city of Bhubaneswar, Odisha, India (20.27° N, 85.84° E) and kept in enamel coated trays (45×60cm) containing 4 to 6 cm deep conditioned tap water (tap water stored and aerated for 72 hours). After hatching, rearing of tadpoles was done following standardized procedure (Mohanty-Hejmadi, 1977). The tadpoles were fed with boiled *Amaranthus* leaves *ad libitum*.

Tail amputation and vitamin A / retinoic acid treatment

Tadpoles of Gosner (1960) stages 26-28 (hind limb bud stage) were selected for tail amputation. The tadpoles were anaesthetized in 1:3000 solution of MS222 prior to amputation through the middle of the tail by keeping them laterally on a pre-sterilized porcelain plate. After operation, the tadpoles were transferred to amphibian ringer solution for about 10 minutes to prevent further loss of blood. Tadpoles of the control group were reared in conditioned tap water where as the tadpoles of the experimental group were treated with 10 to 30 IU/ml of vitamin A palmitate for different time periods ranging from 24 to 144 hours. In case of retinoic acid treatment, tadpoles were exposed to concentration of 125ng/ml to 750ng/ml of retinoic acid for 24 to 72 hours. Required amount of Vitamin A or retinoic acid was added to conditioned tap water in separate glass troughs (1000ml capacity) to make the volume 500ml where five tail amputated tadpoles were reared (optimum rearing condition). Following treatment they were transferred to conditioned tap water keeping the volume same to avoid effect of crowding. Tail amputated tadpoles of both control and treated

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groups were kept under observation till onset of metamorphosis i.e., emergence of fore limbs or death following which they were fixed in 4% buffered formalin for further morphological investigation.

Differential staining of bone and cartilage

Standard Alizarine red and Alcian blue stain technique was used for bone and cartilage differentiation (Wassersug, 1976). Tadpoles selected for staining ectopic limb bones were anesthetized with MS222 and fixed in 4% formalin for 48 hours. Skin of the fixed tadpoles was removed along with other soft organs including the eyes. Tadpoles were washed in gently trickling tap water approximately for 12 hours followed by 3-4 times wash with distilled water. Before staining the cartilage, excess water was blotted off from the body surface. The tadpoles were then stained with alcian blue solution (60ml absolute ethyl alcohol + 40ml glacial acetic acid + 9mg alcian blue 8Gx powder) in an air tight container for 24-48 hours. The tadpoles were drained and blotted off before transferring to absolute ethyl alcohol which was changed at 24 hours interval for 3-4 days. Dehydration was done carefully to avoid fading of alcian blue stain. Following complete dehydration, the tadpoles were blotted and placed in 100ml of 0.5% potassium hydroxide solution to which 3-4 drops of 0.1% alizarin red S solution was added for staining bones. Maceration was allowed until the specimen became transparent. Clearing was completed by transferring the stained tadpoles to a graded series of glycerol-water solution (25%, 50% and 75%). Finally the tadpoles were transferred to 100% glycerol in an air tight transparent container to which a small crystal of phenol was added to retard any spoilage. The stained tadpoles were viewed under microscope to assess the development of bone (red) and cartilage (blue).

Histology and immunohistochemistry

For histology and immunohistochemistry, tails were fixed in 4% buffered formalin for 5-6 hours, washed overnight, dehydrated with ascending series of ethanol and then embedded in paraffin wax (56-58°C). Five μm thick transverse sections (TS) and longitudinal sections (LS) were cut as per requirement using a rotary microtome. For histology, Mallory's triple staining method was followed using acid fuschin, phosphomolybdic acid, aniline blue and orange G.

For immunohistochemical localisation of Acid phosphatase, tissue sections deparaffinised with xylene were rehydrated with descending series of ethanol. Sections were then treated with antigen retrieval solution (50 mM glycine-HCl, pH 3.5) maintained at 95°C for 5 minutes to unmask the antigens. After cooling to room temperature, the tissue sections were first rinsed gently with deionised water followed by 1× Phosphate buffered saline (PBS), pH 7.4. The sections were then incubated with peroxidase blocking reagent (3% H_2O_2) for 10 minutes to quench endogenous peroxidase activity. To reduce non-specific hydrophobic interactions, the sections were blocked with serum blocking reagent for 15 minutes and incubated with avidin and biotin blocking reagents for 15 minutes each to block binding of secondary reagents to endogenous biotin and avidin. The sections were incubated overnight at 4°C with 2 μg/ml concentration of primary antibody in incubation buffer. The negative controls were also incubated overnight but in non-immune serum. The sections were further incubated with anti-sheep biotinylated secondary antibody for 30 minutes and then with high sensitivity

Fig. 1. Development of ectopic limb along with regeneration of dorsal tail fin in tadpoles of *Polypedates maculatus* *with 500 ng RA/ml treatment for 72hours following tail amputation through mid of the tail, (Scale bar, 2 mm).*

streptavidin conjugated to horseradish peroxidase (HSS-HRP) for 30 minutes. Visualization of bound primary antibodies was achieved by incubation with 3, 3′ Diaminobenzidine (DAB) for 10 minutes. Sections were counterstained with 1% haematoxylin for 1 minute. The slides were finally rinsed with absolute alcohol, cleared with xylene and mounted with DPX. All steps were performed at room temperature unless otherwise specified (R&D Systems, U.S.A). Photographs of whole tails were taken using a Leica MZ6 stereomicroscope and Pentax K1000 camera. For immunostained and their corresponding Mallory sections, a Hund, H500 compound brightfield microscope and a microscope eyepiece digital camera, CatCam130 were employed (Mahapatra *et al.,* 2017).

Immunofluorescence localization

For immunofluorescence localisation, tissue sections of 5μ thickness were collected on pre-coated gelatinized slides and immunofluorescence labelling was performed in the subsequent days according to the instructor's manual (Santa Cruz Biotechnology, U.S.A.). The slides were incubated in 10% normal donkey serum, diluted in phosphate buffered saline (PBS) for 30 min. Goat Polyclonal IgG specific for FGF was diluted (1:50) in 1.5% antibody diluent (Normal donkey serum- PBS) and applied on the tissue sections. The samples were incubated overnight in 4 °C inside a humidifying chamber. After repeated rinse in PBS, the samples were once again incubated in anti-Goat Donkey-FITC conjugated secondary antibody diluted (1:200) with 2% antibody diluent (Normal donkey serum-PBS) for 30–40 min. The secondary antibody treatment was carried out in a dark chamber. Finally, the sections were mounted using Ultracruz mounting medium. Images were captured with the same exposure and processed similarly using Leica DFC450 C fitted to Leica DM 3000 LED microscope (Hota *et al.,* 2018).

Morphological studies

Normal looking tails regenerated in the control tadpoles within 15 days of tail amputation while abnormal tails regenerated in different time periods depending on the species. In the tadpoles of *P. maculatus*, various types of tail abnormalities appeared (Mahapatra and Mohanty-Hejmadi, 1994). However, recently we have observed (unpublished data) tail abnormalities not evident earlier such as

development of ectopic limbs along with dorsal tail fin (Fig. 1). In more than 20% abnormal tails, bud like structures appeared which subsequently developed into ectopic hind limbs.

Differential staining of bone and cartilage of ectopic limbs

Ectopic limbs were always hind limbs with distinct thigh, shank, ankle and five digits. Generally the ectopic hind limbs were smaller than the normal hind limbs and developed in pairs. In more than 50% cases ectopic limbs originated from distinct pelvic girdles (Fig. 2).

Histology of regenerated tails

Marked histological similarities were reported to exist between normal and vitamin A induced ectopic limb buds in the tadpoles of the Indian tree frog, *P. maculatus* (Mahapatra *et al.,* 2004). However, close association of nephric tubules and lateral plate mesoderm, as seen in normal hind limb bud did not seem to be essential for ectopic limb development (Fig. 3). Scanning electron microscopic studies (Mohanty-Hejmadi and Crawford, 2003) revealed enlargement of notochord in the treated tail regenerates and there was accumulation of yolk platelets at the amputation site (Fig. 4).
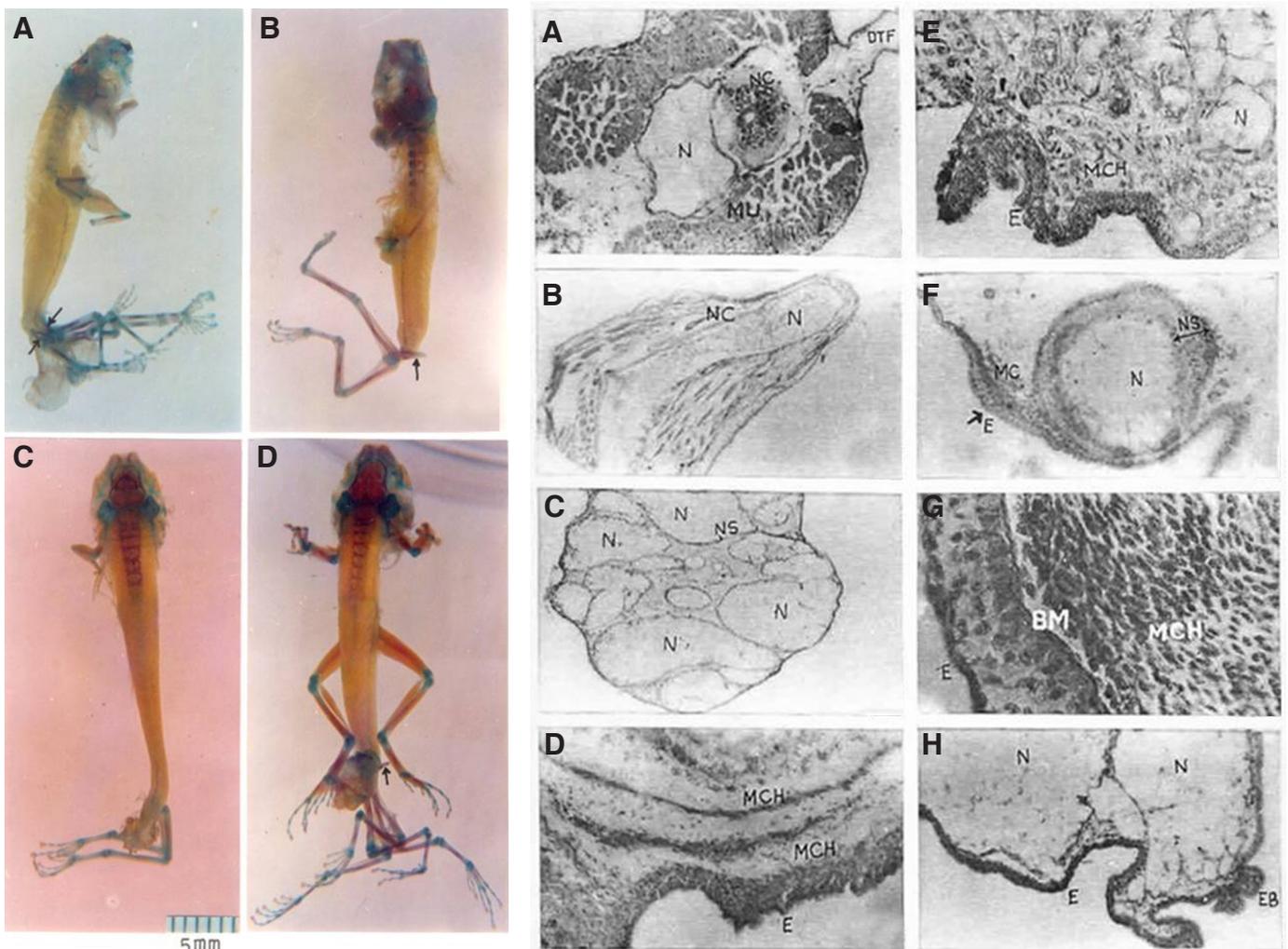
Fig. 2 (Left). Ectopic hind limbs in *Polypedates maculatus* showed both bony (red) and cartilaginous (blue) elements (A-D). *Pelvic girdles indicated as black arrows* **(A,B,D)**. *(Mahapatra, 1994).*

Fig. 3 (Right). Section through the regenerated tails of the control and vitamin A treated tadpoles of *Polypedates maculatus*. (A) *Transverse section through tail of a control tadpole showing notochord (N) spinal cord (SC) and muscle bundle (MU);* **(B)** *Transverse section through vitamin A treated regenerated tail, showing enlarged notochord (N) and small nerve cord (NC);* **(C)** *Transverse section through tail, showing notochordal mass (N) in globules surrounded by notochordal sheath (NS);* **(D)** *Longitudinal section through tail, showing folded epidermis (E) and two layers of condensed mesenchymal cells (MCH) below the epidermis;* **(E)** *Longitudinal section through tail, showing thick epidermis (E), mesemchymal cells (MCH), compact and small globules of notochordal cells (N);* **(F)** *Longitudinal section through abnormal tail, showing folded epidermal layer (E), large notochord (N) with thick notochordal sheath (NS) and accumulation of mesodermal cells (MC) beneath the ectoderm (Arrow);* **(G)** *Longitudinal section through tail, showing thick epidermal layer (E), dark basement membrane (BM) and inner mesenchymal cells (MHC) with intercellular space;* **(H)** *Longitudinal section through abnormal tail, showing vacuolated notochordal cells (N) and two ectopic limb buds (EB) protruding from the epidermal layer (E). (Mahapatra* et al., *2004).*
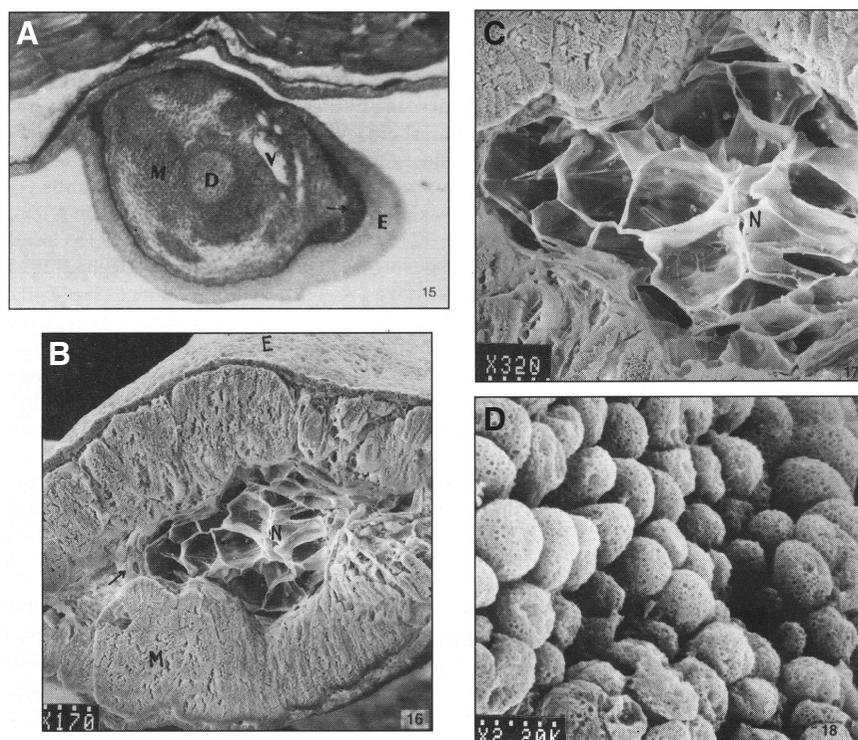
Fig. 4. Section through ectopic limb and amputated tail of the vitamin A treated tadpoles of *Polypedates maculatus*. (A) *Longitudinal section of the ectopic hind limb (EHL) (treated) 48d postamputation. The developing ectopic limb consists of a central core of differentiating cartilage (D), surrounded by differentiating muscle fibres (M) with intervening blood vessels (V). The whole limb is enveloped by a thick epidermis (E) with a thick basement membrane (arrow);* **(B)** *Scanning electron micrograph (SEM) of the tail proximal to the level of amputation after vitamin A treatment 14 d postamputation. Section showing notochord (N), nerve cord (arrow), muscle (M) and the thick epidermis (E);* **(C)** *SEM of the notochord (N) only in higher magnification. The individual chorda cells are enlarged and thin walled;* **(D)** *SEM of tail showing yolk platelets. (Mohanty-Hejmadi and Crawford, 2003).*

Biochemical investigation

Oxygen derived free radicals or oxidants, product of oxygen metabolism, are known to be one of the causative factors underlying development and differentiation as well as very important mediators of cell injury and death. Since cell injury and death are an integral part of amputation essential for regeneration, it was necessary to study role of oxidants during regeneration. In response to oxidants several cellular antioxidants are produced to prevent the organism from oxidative stress. In order to investigate role of oxidative stress (both oxidants and antioxidants) during tail regeneration and vitamin A mediated homeotic transformation, tadpoles of the Indian tree frog, *P. maculatus* were selected because of prolific reaction of this species to vitamin A treatment. Parameters investigated included Lipid peroxidation (LPX), the index of oxidative stress; Hydrogen peroxide (H_2O_2), a potent oxidant; superoxide dismutase (SOD), an enzymatic antioxidant and catalase (CAT), also an enzymatic antioxidant. Besides, reduced glutathione (GSH), a non enzymatic antioxidant normally expressed during cell division was estimated. A higher level of oxidative stress in the regenerating tails was evident as compared to the normal tail and in the treated tail regenerates, the level of oxidative stress remained at the maximum level. Thus, it was established that a hyper-oxidative stress condition prevailed in the abnormal tails, a pre requisite for ectopic limb development (Mahapatra *et al.,* 2002).

Immunohistochemistry - acid phosphatase

The lysosomal marker enzyme, acid phosphatase is known to be expressed by tissue forming precursor cells and has also been reported during regeneration in different groups of animals. Moreover, this enzyme has been described to mediate the process of dedifferentiation (Mahapatra *et al.,* 2017). In an attempt to evaluate

the role of acid phosphatase expressing cells during vitamin A induced abnormal tail regeneration, a prerequisite for ectopic organ formation, tadpoles of *P. maculatus* were considered because of their response to vitamin A.

In the non-amputated tails, acid phosphatase was mainly restricted to the epidermis and muscle patches. In the tail regenerates of the control group, notochordal sheath, spinal cord along with epidermis and muscle patches stained for this enzyme. In vitamin A treated tails, acid phosphatase was mostly localized in the epidermis, notochord precursor cells and undifferentiated cells of the mesenchyme. As, acid phosphatase was mainly expressed by tissue forming precursor cells, this enzyme seems to be involved in tissue remodelling processes (Mahapatra *et al.,* 2017).

Role of fibroblast growth factors during spinal cord regeneration

Fibroblast growth factors (FGFs) are mitogenic and neurotrophic factors and are well known for their diversified roles during proliferation and wound healing processes. Our laboratory has demonstrated by immunofluorescence localization the involvement of Fibroblast Growth Factor1 (FGF1) during spinal cord regeneration in anurans for the first time (Hota *et al.,* 2018). Spinal cord during tail regeneration in *P. maculatus* attains functional recovery within a span of 2 weeks thus enabling the organism to survive in an aquatic medium till metamorphosis. This study on spinal cord regeneration not only provides evidences of FGF1 as a neurotrophic factor that aids in the self-repair of spinal cord (Fig. 5) but also support the progenitor mediated tail regeneration model in anurans as has been proposed earlier (Hota *et al.,* 2018).

Conclusion

In summing up the phenomenon of homeotic transformation, as reported by Mohanty Hejmadi and Crawford (2003) and Mahapatra *et al.,* (2004 and 2017), morphological, skeletal and histological studies indicate that the ectopic limbs at tail are comparable to the normal hind limbs although they develop away from the tissues normally associated with hind limb development. It is believed that vitamin A respecifies the tail tissues into limb tissue as proposed

by Bryant and Gardiner (1992) and supported by the development of axial skeleton during the development of homeotic hind limbs (Muller *et. al*. 1996).

However, recently, Tazawa and Yaoita (2017) have reported the induction of homeotic transformation in Japanese brown frogs, *Rana japonica*, *R. ornativentris* and *R. kobai* tadpoles. Since *axial elements were not observed* and the ectopic limbs developed *not only* at the ventral side *but also* at the dorsal side of the tail; they have proposed an alternative hypothesis to that of Bryant and Gardiner (1992). Considering that ectopic limbs arose from tail regenerates with insufficient fin regeneration, they have speculated that *fin-forming cells* may modulate and contribute to the ectopic

Fig. 5. Spinal cord regeneration in amputated tail of *Polypedates maculatus* (A) *Transverse section of normal spinal cord, Mallory's triple stained;* **(B–D)** *Longitudinal section of 2, 3 and 4 day regenerates stained with Mallory's triple stain;* **(E)** *FGF1 immunostaining of normal spinal cord;* **(F–H)** *FGF1 immunofluorescence detection in 2, 3 and 4 day regenerates, respectively. (Scale bar, 25 μm). (ep, ependymal cells; m, melanocytes; ms, mesenchymal cells; na, neural ampulla; nc, neural canal; arrowheads, cluster of cells near the cut site) (Hota* et al., *2018).*

limb formation. Based on the sites of ectopic limb emergence, Morioka *et al.,* (2018); have supported this view that fin cells may contribute to the limb formation.

In the most extensive and recent review, giving a detailed account of the data available now for the last 25 years on homeotic transformation, Morioka *et al.,* (2018) have discussed the key issues which need to be addressed at present. As indicated by them, inspite of *the extensive information gained from morphological observations,* a *comprehensive understanding of this phenomenon is essential at molecular level.*

It is pertinent to mention that in comparison to the anurans, regenerating capacity of urodeles at cellular and molecular levels are well known (Geraudie and Ferretti,1998). Growth is controlled in the regeneration blastema at cellular and molecular level. They have also discussed about molecules that are likely to be involved in regenerating limb such as homeobox genes and retinoids. Further as indicated by Slack and Tannahill (1992) defects in the axial pattern may be produced by retinoic acid but it remains unclear whether its effects are coordinate ones or are concentrated in certain regions of high sensitivity. It is necessary to explore these aspects during the *the homeotic transformation in anurans.*
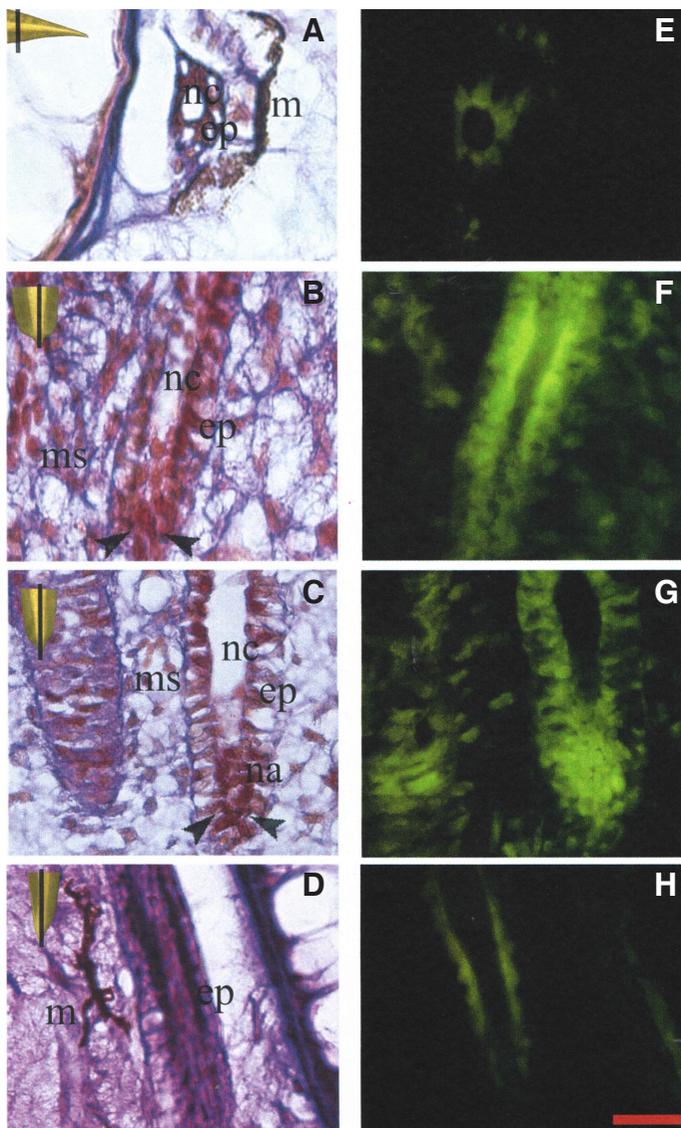
Keeping this in mind, Morioka and his group at present, are analysing the phenomenon at molecular level. They have indicated that it is now possible to clone genes and genome fragments utilizing published sequence data for *Xenopus tropicalis* and *X. laevis,* examine their expression profiles by RT-PCR, and assay their functions using genome editing techniques in non-model organisms (see p.374; Morioka *et al.,* 2018). Their studies will be of immense help to understand this unique phenomenon.

Last but not the least, all of the regenerated tissues (including cartilage, muscle, and nerves) are derived from this cellular aggregate. There are two schools of thought: axolotls (as well as other animals that regenerate tissues) have an endless supply of stem cells that contribute to tissue regeneration. Alternatively, the act of wounding induces cells within each tissue compartment to reverse to a more stem-like state, through a process called de-differentiation, and these cells are responsible for regenerating the missing limb (Zhao and Helms, 2011).

In our study, it was observed that the movement of the cells at the site of amputation was very quick and their concentration varied. The whole limb was generated indicating that there must be a reserve of stem cells and *cue* to positional information to re-differentiate. The enlargement of the notochord and concentration of yolk platelets is interesting. Whether they actively contribute to de-differentiation has to be determined in future study. As indicated earlier, Morioka *et al.,* (2018) are already examining the contribution of fin cells to the limb formation.

It has been reported that vertebrate hox genes are regulated by retinoid (Huang *et al.,* 2002; Marshall *et al.,* 1996) and *Hox* genes encode transcription factors that play a key role in specifying the body plan in metazoans (Mannaert *et al.,* 2005). Possible role of vitamin A / retinoic acid in expressing hindlimb specific hox genes during homeotic transformation of tail to hindlimbs in anuran tadpoles is suggested and therefore, has to be pursued in future investigations.

At present our laboratory is investigating the possible role of fibroblast growth factors (FGFs) as candidates mediating homeotic transformation in the Indian tree frog, *P. maculatus* as a suitable model from Indian context (Hota and Mahapatra, 2017; 2018a,b).

We are also in the process of Genome sequencing of this species which may unfold molecular mechanism of transdifferentiation of cells during homeotic transformation.

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