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Hyperinnervation improves *Xenopus laevis* limb regeneration

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A B S T R A C T

Xenopus laevis (an anuran amphibian) shows limb regeneration ability between that of urodele amphibians and that of amniotes. *Xenopus* frogs can initiate limb regeneration but fail to form patterned limbs. Regenerated limbs mainly consist of cone-shaped cartilage without any joints or branches. These pattern defects are thought to be caused by loss of proper expressions of patterning-related genes. This study shows that hyperinnervation surgery resulted in the induction of a branching regenerate. The hyperinnervated blastema allows the identification and functional analysis of the molecules controlling this patterning of limb regeneration. This paper focuses on the nerve affects to improve *Xenopus* limb patterning ability during regeneration. The nerve molecules, which regulate limb patterning, were also investigated. Blastemas grown in a hyperinnervated forelimb upregulate limb patterning-related genes (*shh, lmx1b,* and *hoxa13*). Nerves projecting their axons to limbs express some growth factors (*bmp7, fgf2, fgf8,* and *shh*). Inputs of these factors to a blastema upregulated some limb patterning-related genes and resulted in changes in the cartilage patterns in the regenerates. These results indicate that additional nerve factors enhance *Xenopus* limb patterning-related gene expressions and limb regeneration ability, and that *bmp, fgf,* and *shh* are candidate nerve substitute factors.

1. Introduction

Xenopus laevis froglets cannot regenerate their limbs completely (Dent, 1962; Suzuki et al., 2006; Yokoyama, 2008). *Xenopus* tadpoles, on the other hand, can completely regenerate their limb buds in the early stages of development although this regeneration ability gradually declines as they progress through advancing developmental stages (Dent, 1962; Nieuwkoop and Faber, 1956). After limb bud amputation, a mass of undifferentiated cells called a blastema is induced on the amputation plane. The blastema forms a limb in a manner similar to that of a developing limb bud. Postmetamorphic frogs retain the limb regeneration ability, but the regenerate becomes hypomorphic. Limb amputation of a froglet results in a cone-shaped cartilaginous structure called a "spike" (Dent, 1962; Suzuki et al., 2006). The spike has neither joints nor branches. Morphological and tissue defects, such as a lack of muscles, have also been reported (Dent, 1962; Endo et al., 2000; Satoh et al., 2005). Attempts to improve such defects in *Xenopus* froglet limb regeneration have been reported. Chemical treatments, repeats of limb amputation, additional nerve supply, and transplant of limb bud mesenchyme were reported to enhance limb patterning ability of froglet limb regeneration (Scadding and Maden, 1986; Bernardini et al., 1996; Cecil and Tassava, 1986; Kurabuchi, 1992; Tsilfidis and Liversage, 1989; Lin et al., 2013). Yet, perfect limb regeneration in a *Xenopus* froglet has not yet been achieved.

Generally, the limb regeneration process can be divided into three characteristic series of steps, namely, wound healing, blastema induction, and pattern forming. In the wound healing process, the amputation surface is covered with epithelial cells in an epithelial structure called the "wound epithelium" (Carlson et al., 1998; Suzuki et al., 2005, 2006). The wound healing process can be seen in a regeneration incompetent animal. Blastema induction depends on the presence of nerves and nerve-dependent blastema formation is necessary for successful limb regeneration in amphibian limb regeneration (Singer, 1951, 1974; Endo et al., 2000; Suzuki et al., 2005; Brockes, 1987; Yokoyama et al., 2011; Kumar and Brockes, 2012; Korneluk et al., 1982). The identification of the involved nerve factors has been a major theme in amphibian limb regeneration since regeneration-incompetent animals cannot undergo blastema induction. Various candidate genes have been suggested, including Ggf (glial growth factor), nAG (newt anterior gradient), neuregulin, Fgf (fibroblast growth factor), and Bmp (bone morphogenic protein) (Brockes and Kinter, 1986, Kumar et al., 2007; Farkas et al., 2016; Mullen et al., 1996; Makanae et al., 2016, Satoh et al., 2015). Our previous studies demonstrated that *fgf* and *bmp* genes can be substituted for nerves in blastema induction in multiple species and organs including *Xenopus* froglets (Satoh et al., 2015; Makanae et al., 2016, 2014). Thus, nerve molecules and functions in the blastema induction phase are beginning to be

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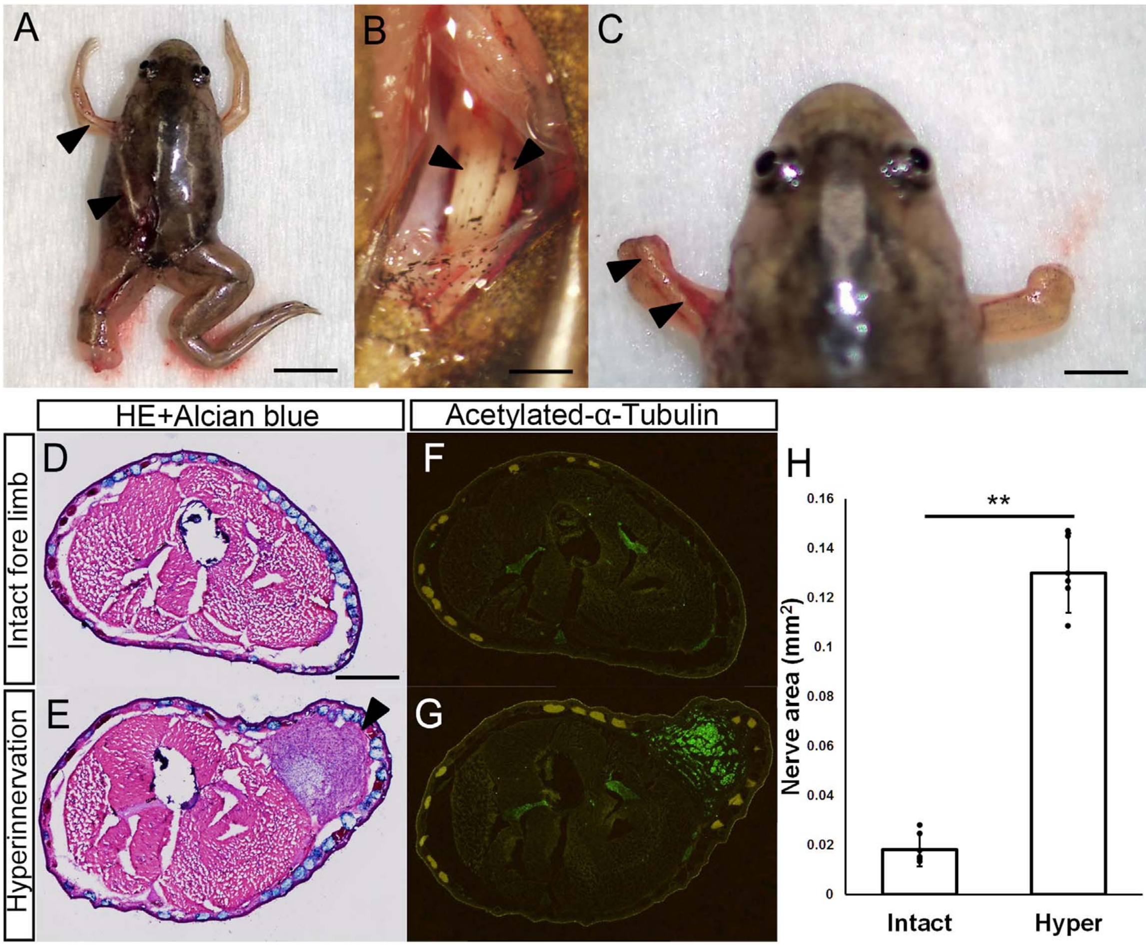

Fig. 1. Hyperinnervation of forelimb from hind limb (A–C) The scheme of nerve deviation and rerouting to forelimb. Arrow heads indicate neurons projecting to a hind limb. (D, E) Alcian blue and, haematoxylin and eosin staining of an intact limb and a hyperinnervated limb. (F, G) Neural cells (alpha acetylated tubulin) were visualized by immunofluorescence. (H) Calculated nerve amounts in an intact limb and a hyperinnervated limb by alpha acetylated tubulin positive pixels. Intact: intact fore limb and Hyper: hyperinnervated limb. ** $p < 0.01$ (Welch's *t*-test, n = 6). Bars represent standard deviation. Scale bars in A, B, C, and D are 5, 1, 2, and 0.2 mm, respectively. D–G are the same magnification.

understood. Once a regeneration blastema has formed, it is considered to mimic limb developmental processes to form a patterned limb, which is the pattern forming stage. Especially, nerve roles in the pattern-forming stages remain widely unknown. In urodele amphibians, a blastema that was denervated in the pattern-forming stage resulted in miniature limbs with complete digits (Stocum, 2011). Previous studies have also demonstrated the relationship between pattern formation and nerves in *Xenopus* froglet limb regeneration (Konieczna-Marczynska and Skowron-Cendrzak, 1958; Kurabuchi, 1992; Kurabuchi and Inoue, 1983). In *Xenopus* froglet limb regeneration, a blastema that was denervated after blastema formation stages result in significantly regression of regeneration (Kurabuchi and Inoue, 1983). Hyperinnervation from a hind limb to a forelimb resulted in a branched structure in *Xenopus* froglet limb regeneration (Konieczna-Marczynska and Skowron-Cendrzak, 1958; Kurabuchi, 1992). However, the molecular mechanism of branch formation by hyperinnervation is unknown.

In this study, hyperinnervation experiments in a *Xenopus* froglet blastema were revalidated, and the positive effects of hyperinnervation on limb patterning were investigated. In limb regeneration in regeneration competent animals, *shh*, *lmx1b,* and *hoxa13* genes are expressed in the posterior, dorsal and distal regions of a regeneration blastema (Ohgo et al., 2010; Matsuda et al., 2001; Endo et al., 2000; Yakushiji et al., 2007). However, *shh*, *lmx1b,* and *hoxa13* genes are generally not properly upregulated during *Xenopus* froglet limb regeneration (Ohgo et al., 2010; Matsuda et al., 2001; Endo et al., 2000; Yakushiji et al., 2007). We found that these gene expressions were improved by hyperinnervation. To investigate nerve regulation in *Xenopus* froglet limb regeneration, we focused on *Shh*, *Fgf* and *Bmp* genes. Our previous study clearly demonstrated that *Fgf* and *Bmp* genes are expressed in amphibian dorsal root ganglion (DRG) neurons (Makanae et al., 2014; Satoh et al., 2015). We also found *shh* expression in *Xenopus* DRG. We investigated the effects of those factors by *in-vitro* and *in-vivo* assay and found that *bmp+fgf (+shh)* gene affected *Xenopus* froglet blastemas with regard to limb patterning-related genes and cartilage morphology. Investigating hyperinnervation effects in blastemas enables an assay of the endogenous nervous factors necessary for pattern formation improvement. Additionally, determining whether *bmp+fgf (+shh)* can act as nerve factors in pattern formation is important for application to amphibian studies on limb regeneration in higher vertebrates. Our findings improve the understanding of limb patterning in limb regeneration processes controlled by neural regulation.

2. Results

2.1. Hyperinnervated blastema formed a branched structure

To examine nerve effects on limb patterning in *Xenopus* limb regeneration, nerve bundles projecting to a *Xenopus* froglet hind limb were rerouted to a forelimb as shown in Fig. 1A and B. Amputation was performed at the mid-zeugopod level two weeks after the hyperinnervation surgery (Fig. 1C). Even though each operated froglet lost three limbs, each was able to feed and swim (Sup. movie 1). The procedures