Title of Thesis

The role of nerves in *Xenopus laevis* froglets’ limb regeneration

アフリカツメガエル四肢再生における神経の役割

2018, March

Kazumasa Mitogawa

Graduate School of Natural Science and Technology

(Doctor’s Course)

OKAYAMA UNIVERSITY
Abstract

*Xenopus laevis* (an anuran amphibian) shows unique limb regeneration ability between that of urodele amphibians and amniotes. *Xenopus* froglets can initiate limb regeneration processes but fail to form patterned limbs. Regenerated limbs mainly consist of a 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. It was found that hyperinnervation to an amputated limb improved the pattern defects in a regenerate, resulting in the induction of a branching regenerate. The hyperinnervation in a *Xenopus* limb allows the identification and functional analysis of the molecules controlling this patterning of limb regeneration. This paper focuses on the nerve affects improving *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.
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General Introduction

Regeneration in animals

Regeneration is one of the most general abilities among living things and can be observed in many species. Regeneration ability has fascinated scientists and been a major research target for a long time (Ikeuchi et al., 2016; Umesono et al., 2013; Hamada et al., 2015; Kang et al., 2016; Sugiura et al., 2016; Simkin et al., 2017). Regeneration in plants and flatworms occasionally function as a form of reproduction because members of these groups can regenerate additional bodies from the severed tips of their original bodies. Some fishes and urodele amphibians are also recognized as exceptional regenerators as they can regenerate many organs, including the limbs, the skin, the tail, the fins, the heart, the brain and the lens, though they cannot regenerate a whole body from parts (Mitogawa et al., 2015; Fei et al., 2014; Tornini et al., 2017; Tanaka et al., 2016; Sousounis et al., 2014; Seifert et al., 2012a; Eguchi et al., 2011; Samches et al., 2017; Kang et al., 2016; Maden et al., 2013). Mammals such as mice and humans, on the other hand, generally cannot regenerate organs as fishes and urodele amphibians can. On the contrary, mammals have very limited regeneration ability as represented by digit tip regeneration (Takeo et al., 2013; Gao et al., 2013; Seifert et al., 2012b; Hardy et al., 2016). Even humans can regenerate the extreme distal tips of digits after amputation (Shieh and Cheng, 2015), however such regeneration ability is limited to the very ends of the distal phalanges. It is considered that mammals lost the capacity for organ-level regeneration during the course of evolution. To understand which genetic systems were silenced down during the evolutionary process resulting in the loss of regeneration ability, a comparative analysis between regeneration-competent and
-incompetent animals is necessary and has been one of the biggest themes in regenerative biology.

**Limb regeneration in amphibians**

Limb regeneration has been investigated as the representative phenomenon of the powerful regeneration ability that can be observed in urodele amphibians. An axolotl (*Ambystoma mexicanum*) can regenerate a limb morphologically within four weeks after amputation, although another one or two months are needed to make it fully functional (Makanae and Satoh, 2012; Fig. A). The amphibian limb regeneration process is conceptually divided into three steps; 1) wound healing, 2) blastema formation, and 3) pattern formation (Endo et al., 2004). Investigation of the differences at each regulatory step between regenerative and non-regenerative animals will enable us to understand regeneration ability throughout the body, since a common regulatory system is thought to regulate regeneration processes in many organs.

The first step, the wound healing process, begins immediately after limb amputation. Amputation creates an exposed surface in the limb which must be covered by the surrounding epidermis. Epidermal cells near the amputation plane start migrating to cover the exposed surface. This migrating epidermis is called the wound epidermis/epithelium (WE) (Carlson et al., 1998; Suzuki et al., 2005; Suzuki et al., 2006). The WE migration process appears to be mostly conserved from anuran amphibians to mammals. The major difference between the classes is that the migration velocity of the WE is much faster in amphibians than in mammals (Satoh et al., 2008; Rittié, 2016). In animals with no limb regeneration ability, the second step is not induced, and the tissue proceeds directly to dermis reconstitution and thence to skin
wound healing. At this point, regeneration-incompetent animals including adult mice and humans form scar tissue that lacks skin appendages, such as hair follicles (Bertolotti et al., 2013; Takeo et al., 2015; Kawasumi et al., 2013). In regeneration-competent animals such as amphibians, on the other hand, the thick collagen layer is not formed, and dermis reconstruction does not occur (Satoh et al., 2008). Thus there is a clear distinction between amphibians and mammals in the wound healing process.

In the progression of regeneration from the first step to the second step, the nerves play an essential role. Blastema formation, the primary phenomenon in the second step, requires the presence of nerves. Neural axons projecting from the dorsal root ganglia (DRG) to the limbs innervate the wounded skin; this process, is considered to induce the specification of WE into regeneration-specific epidermis (Bryant et al., 2002). The thin layer of epithelial cells thickens into multilayered WE, which is called the apical epithelial cap (AEC). The AEC promotes limb regeneration by interacting with the underlying connective tissues to gather undifferentiated cells and form them into a blastema. This interaction between nerves and WE is considered a primary force behind the induction of a blastema at the amputated plane (Fig. A). If the nerves are dissected out from the limb before blastema formation, no blastema is induced and no regeneration occurs. Therefore, nerves must be present for blastema formation to occur.

The third step in limb regeneration is the pattern formation step. In this process, the blastema cells redifferentiate in order to reconstitute the pattern of the lost limb. The blastema expresses patterning and differentiation factor genes comparable to those seen in a developing limb bud (Muneoka and Bryant, 1982). Like the blastema formation step, the pattern formation step is also thought to be dependent on the nerves. The nerves have been suggested to be involved in the maintenance of blastema cell
proliferation, given that, in the axolotl (*Ambystoma mexicanum*), a blastema denervated after blastema induction results in a miniature limb (Stocum, 2011). Yet the nerves may also play a role in limb pattern formation, given that, when nerves are dissected out from a limb in the early blastema stages, the blastema fails to form any structures, resulting in regression. The details of the nerves’ role in the pattern formation step is still largely unknown.

These three regeneration steps and the dependency of the processes on the nerves are the major principals of regeneration. The same regulatory steps can be seen in the regeneration of other organs. Regeneration of a tail, for example, proceeds in a manner similar to limb regeneration. Similarly, dependence on the nerves for regeneration is also seen in the tail, as blastema induction in the tail is also dependent on nerve (spinal cord) presence. If the spinal cord is dissected out from the tail before blastema formation, no blastema is induced and regeneration does not occur. Additionally, relocation of the spinal cord can influence tail pattern formation. Therefore, understanding the roles of the nervous system in the limb regeneration process and in each of its three steps will enable us to understand organ regeneration in amphibians.

**Comparative analysis of regenerative-competent and -incompetent animals**

The African clawed froglet (*Xenopus laevis*), an anuran amphibian, cannot regenerate its limbs completely (Fig. A). Rather, the amputation and healing process results in a cone-shaped structure called a spike, which mainly consists of a single cone-shaped piece of cartilage and contains neither muscle nor joints (Fig. B). Several studies of the regulatory steps in spike formation have been published to date. In the first step, wound
healing, the *Xenopus* froglet can regenerate its skin completely whereas the adult cannot (Yokoyama et al., 2011; Bertolotti et al., 2013). The second step, blastema induction, is nerve-dependent in *Xenopus* as it is in amphibians (Endo et al., 2000). A partially denervated limb results in a decreased rate of successful blastema induction (Yokoyama et al., 2011b). Furthermore, the roles of the nerves in blastema induction were shown to be equivalent between *Xenopus* and axolotls in a unique experimental system called the accessory limb model (Endo et al., 2004; Mitogawa et al., 2014; Satoh et al., 2015; Fig. C). In the third step, pattern formation, the *Xenopus* froglet blastema lacks pattern formation gene expressions and shows no sign of patterning (Suzuki et al., 2006; Mitogawa et al., 2014; Yakushiji et al., 2007; Matsuda et al., 2001; Ohgo et al., 2010). Thus, the third step shows the largest difference between the partial regenerative process in *Xenopus* and the complete regenerative process in the axolotl.

Several studies have suggested that the pattern defect in the *Xenopus* blastema is caused by the lack of gene expression (Yakushiji et al., 2007; Matsuda et al., 2001; Ohgo et al., 2010; Yakushiji et al., 2009). To enhance the patterning ability in *Xenopus* limb regeneration, various treatments including such as limb bud cell transplants and hyperinnervation have been attempted (Lin et al., 2013; Satoh et al., 2017; Konieczna-Marczynska and Skowron-Cendrzak, 1958; Kurabuchi, 1992). A blastema containing limb bud cells results in a digit-like structure rather than a cone-shaped spike (Lin et al., 2013; Satoh et al., 2017; Fig. C). Hyperinnervation from a hind limb to a forelimb, meanwhile, results in a branched structure (Konieczna-Marczynska and Skowron-Cendrzak, 1958; Kurabuchi, 1992; Fig. C). In spite of these efforts, the molecular mechanism for enhancement of pattern formation is unknown. Here, I investigated the role of the nerves in the pattern formation step of *Xenopus* limb regeneration in order
to clarify the mechanisms by which regeneration ability can be enhanced (Mitogawa et al., 2018).

In mice, several defects have been reported in each step of limb regeneration, however, hypotheses have been proposed regarding ways to overcome these defects. In mice, the wound healing step involves scar formation, which does not occur in regeneration-competent animals. In the second step, nervous axons fail to interact with the wound epidermis, preventing blastema formation (Miura et al., 2015). Exceptionally, however, blastema formation can be observed when only the very tip of a terminal phalanx is amputated (Han and Muneoka, 2011). In mice, a few attempts to improve the defects of regeneration in digit amputation have been reported. For instance, continuous input of bone morphogenic protein 2 (BMP2) or bone morphogenic protein 7 (BMP7) to a wound directs cartilaginous extension from the amputation plane (Ide 2012; Yu et al., 2012). The tissues that have thus been regenerated in mouse digits are single cone-shaped growths similar to the spikes seen in *Xenopus* limb regeneration. Therefore, the investigation of the nerves’ roles in pattern formation in *Xenopus* limb regeneration may lead to important insights regarding higher vertebrates’ limb regeneration. To pursue this possibility, this study investigated ways of improving of pattern formation in *Xenopus* limb regeneration and the molecular mechanisms responsible for these changes. A deeper understanding of how limb patterning controlled by neural regulation is required for the application of amphibian limb regeneration studies to higher vertebrates.
Fig. A: Axolotl and *Xenopus* limb regeneration.

(Upper) An example of axolotl (*Ambystoma mexicanum*) limb regeneration. (Lower) An example of African clawed frog (*Xenopus laevis*) limb regeneration. Both amphibians can form a blastema at the amputated plane within a few weeks after amputation. The axolotl’s blastema can regenerate well-patterned limbs, whereas the *Xenopus* frog’s cannot, generating single cone-shaped patternless structures instead. The difference in regenerative ability is not linked to any difference in the amputation plane. Time scales: before amputation, at amputation, and at one week, two weeks, three weeks, four weeks and five weeks after amputation.
Fig. B: An intact *Xenopus* limb and a regenerated spike.

(Left) The intact limb contains jointed cartilage and muscle. (Right) The regenerated spike under H&E stain. Muscle fibers (MHC) and nerve filament (Acetylated alpha tubulin) are visualized by immunohistochemistry. The intact limb contains muscle fibers and nerves at its distal end. The spike is composed of cartilage and does not contain muscle fibers within the regenerated region. The black line indicates the amputation plane.
Fig. C: An ectopic blastema and enhanced limb blastema phenotypes in *Xenopus* limb regeneration.

(Upper left) An accessory blastema of an axolotl. This ectopic blastema was induced from a nerve and skin wound. (Upper right) An accessory blastema of a *Xenopus* frog. This ectopic blastema was induced from a nerve and skin wound like that in the axolotl. (Lower left) Digit-like structures were induced by the transplantation of limb bud cells to a blastema. (Lower right) Digit-like structures were induced by hyperinnervation surgery.
References


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; Suzuki et al., 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; Endo et al., 2000; Suzuki et al., 2005; Brockes, 1987; Singer, 1974; 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 morphogenetic 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; Makanae et al., 2014). Thus, nerve molecules and functions in the blastema induction phase are beginning to be 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.
Results

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 Figure 1A and 1B. 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 successfully achieved hyperinnervated stimulation as compared to a normal limb (Fig. 1D–H). As a control for the functional sciatic nerve bundle, an amputation of the denervated sciatic nerve in the back was performed two weeks after the hyperinnervation surgery (Table 1). Hyperinnervation with amputated sciatic nerves resulted in spike formation (Table 1). The extra nerve bundle could be seen at the dorsal side of the limb (Fig. 1E, G). The amount of nerve area in the forelimb was measured by α-acetylated tubulin-positive pixels in each amputated plane (Fig. 1H). The morphological changes in the blastema became apparent approximately eight weeks after the surgery, and appeared to be settled by 12 weeks after the surgery. The cartilage pattern was then confirmed by whole-mount Alcian blue and Alizarin red staining (Fig. 2D–F, Table 1). Amputation of control limbs (intact limbs) resulted in spike structures (Fig. 2A, D). Hyperinnervated blastemas generally showed three phenotypes: a spike with a single cartilage cone (data not shown), a spike with multiple cartilaginous structures (Fig. 2B, E), or a branched structure (Fig. 2C, F). In the case of the branched structure, the branching generally appeared from about the wrist level (Fig. 2C, F). To observe the detailed structure, the hyperinnervated blastema was sectioned (Fig. 2H). Histological analysis revealed that the hyperinnervated regenerates had numerous
cartilage clusters whereas the normal spike did not (Fig. 2G, H). Although many such cartilage clusters were formed in the regenerate, there were no signs of joint formation among them. Another feature of the hyperinnervated regenerates was their longer length (Fig. 2I). The hyperinnervated regenerates were larger than the normal regenerates, suggesting that cell proliferation of the hyperinnervated blastema was accelerated. To investigate the mitogenic activity in the hyperinnervated blastema, bromodeoxyuridine (BrdU) labeling to examine the effect of hyperinnervation on cell proliferation was performed two weeks after the amputation (Fig. 2J, K). The number of BrdU-positive blastema cells was calculated according to the BrdU-positive cells/ Hoechst-positive cells in each blastema (Fig. 2L). The hyperinnervated blastemas had a rate of cell proliferation approximately 1.4 times that seen in the control blastema (Fig. 2L). These results indicate that nerves do affect the regulation of limb patterning and blastema cell proliferation.

**Patterning-related genes were upregulated in hyperinnervated blastema**

The branched structure induced by hyperinnervation appeared to have certain directionality. Branching was not random; rather, it always occurred on the anterior-posterior axis (n = 4 of 4). Additionally, branching started at around the wrist level in most cases (n = 3 of 4). This branching pattern, which was consistent with that seen in a previous report (Kurabuchi, 1992) implies that a certain active pattern regulation, rather than mere hyper-cell growth, was working in the hyperinnervated blastema.

To investigate active limb patterning, we examined the gene expression of a hyperinnervated blastema by *in situ* hybridization and quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis (Fig. 3, 4). Considering that branching
was observed from the wrist level and on the anterior-posterior axis, we investigated three genes, *hoxa13*, *lmx1b* and *shh*. *Hoxa13* was expressed in the presumptive autopod region of *Xenopus* limb development and tadpole limb regeneration (Fig. 3A; Endo et al., 2000; Ohgo et al., 2010). In *Xenopus* froglet limb regeneration, *hoxa13* expression was observed throughout the blastema (Fig. 3B; Ohgo et al., 2010). In the hyperinnervated blastema, *hoxa13* expression was more recognizable in the distal region than in the proximal region (Fig. 3C, F). To confirm this relatively distal-restricted *hoxa13* expression, quantitative RT-PCR analysis was performed (Fig. 3G). The control and the hyperinnervated blastemas were separated into, distal and proximal halves, along the length of the blastema for quantitative RT-PCR; two blastemas were used for this experiment. Quantitative RT-PCR analysis revealed that, after hyperinnervation, the *hoxa13* expression level was elevated in the distal half compared to the proximal half (Fig. 3G). Although *hoxa13* expression levels were also higher in the distal half than in the proximal half in the normal blastema, the difference between the expression levels in the two halves was much greater in the hyperinnervated blastema (Fig. 3G).

*Hoxa11* expression has been used as a zeugopod marker in tetrapods and has been confirmed in *Xenopus* limb development (Blanco et al., 1998). *Hoxa11* expression was detected in a regenerating blastema in *Xenopus* (Ohgo et al., 2010). Its expression domain, however, did not appear to be restricted to the presumptive zeugopod (Ohgo et al., 2010). Quantitative RT-PCR analysis revealed that expression of *hoxa11* was not affected by hyperinnervation (Fig. 3H). These results suggest that hyperinnervation enhances distal positional values in the blastema.

*lmx1b* is expressed in the dorsal limb bud mesoderm and plays a central role in
dorsoventral patterning in vertebrate limbs (Riddle et al., 1995). *Lmx1b* was re-expressed in a *Xenopus* tadpole blastema but not in a froglet blastema (Matsuda et al., 2001). Whether hyperinnervation affects *lmx1b* expression was investigated by *in situ* hybridization (Fig. 4). Although *lmx1* expression was detected in the dorsal region of a st. 52 limb bud, it was not detected by *in situ* hybridization in a froglet blastema (Fig. 4A, B). In the hyperinnervated blastema, *lmx1b* expression became detectable by *in situ* hybridization (Fig. 4C, C’). The *lmx1b* expression domain was strictly restricted within a narrow region underneath the blastema epithelium (Fig. 4C’). Large cartilage tissue exists in the center of the blastema, and *lmx1b* expression was not detectable in the cartilaginous domain (Fig. 4C’). Next, we focused on *shh* expression. *Shh* is the gene responsible for anterior/posterior pattern formation in vertebrate limb and fin development (Chiang et al., 2001; Sagai et al., 2005). *Shh* is upregulated in a completely regenerative tadpole blastema but is not re-expressed in a froglet blastema (Endo et al., 2000; Yakushiji et al., 2007). *Shh* expression defect was confirmed in a *Xenopus* froglet blastema (Fig. 4E). A hyperinnervated blastema exhibited a *shh*-positive region on its posterior side (Fig. 4F). Additionally, *smo* and *ptch1*, which are *shh* downstream genes were also upregulated in the hyperinnervated blastema (Suppl. fig. 1). These results demonstrate that hyperinnervation to a limb can reactivate patterning-related genes in *Xenopus* froglet limb regeneration.

**Bmp, fgf, and shh regulate some of nerves’ effects on Xenopus froglet limb regeneration**

Hyperinnervated blastemas resulted in altered morphology and an enhanced yet spatiotemporally appropriate gene expression pattern (Figs. 3, 4). Despite these obvious
changes caused by nerve deviation, the nerve factors involved have not yet been identified. To address this issue, we focused on the nerve factors that have been identified as involved in the blastema induction phases. Our previous studies (Makanae et al., 2016; Satoh et al., 2015) revealed that \textit{bmp7}, \textit{fgf2}, and \textit{fgf8} play nerve roles in blastema induction in amphibians. Furthermore, RT-PCR analysis revealed that \textit{bmp} and \textit{fgf} genes were expressed in \textit{Xenopus} froglet dorsal root ganglion (DRG) (Satoh et al., 2015). These \textit{bmp} and \textit{fgf} expressions in DRG neurons were confirmed by \textit{in situ} hybridization (Fig. 5A). Additionally, \textit{shh} expression was investigated in DRG (Fig. 5A, Suppl. fig. 2), because \textit{shh} plays an essential role in limb development; and because the provision of \textit{shh} signaling enhanced \textit{Xenopus} limb regeneration (Yakushiji et al., 2009, Lin et al., 2013). \textit{Shh} expression was detected with an intense signal (Fig. 5A), suggesting that \textit{shh} is another candidate nerve factor in limb regeneration. Next, whether blastema cells can respond to nerve-expressing factors was examined. \textit{Xenopus} blastema cells cultured with BMP7 had increased phosphorylated SMAD 1/5/8 (pSMAD) (Fig. 5B), whereas those cultured with FGF8 had increased pERK (Fig. 5B). Application of BMP and FGFs onto cultured \textit{Xenopus} blastema cells increased the proliferating cell nuclear antigen (\textit{pcna}) expression levels (Fig. 5C) since hyperinnervated blastemas grow longer regenerates and upregulate cell proliferation. These results suggest that \textit{fgf2}, \textit{fgf8}, \textit{bmp7}, and \textit{shh} are reasonable candidates as nerve factors in \textit{Xenopus} limb regeneration.

Next, the roles of Bmp, Fgf, and Shh signaling in the gene expression pattern of \textit{Xenopus} froglet blastemas were examined (Fig. 6). Hyperinnervated blastemas were grown with a chemical signal inhibitor for each of the examined genes. The hyperinnervated blastemas were either treated with chemical inhibitor for three days or
subjected to denervation were starting 11 days after limb amputation. The inhibitor-treated blastemas were harvested for quantitative RT-PCR. Dorsomorphin, as a Bmp signaling inhibitor, suppressed *hoxa13, lmx1b, ptch1* and *pcna* expression (Fig. 6A). SU5402, as an Fgf signaling inhibitor, significantly suppressed *hoxa13, lmx1b, and pcna* expression, but not *ptch1* expression (Fig. 6A). Cyclopamine, as a Shh signaling inhibitor, also had inhibitory effects on all investigated genes (Fig. 6A). Denervation from the hyperinnervated blastema showed severe suppression of *hoxa13, lmx1b, ptch1*, and *pcna* expression (Fig. 6A). This suggests that multiple nerve factors synchronously regulate those gene expressions. Next, BMP7, FGF2+FGF8 (FGFs), SHH, BMP7+FGFs (BFF), and BMP7+FGFs+SHH (BFFS) protein-soaked beads were grafted into a normal blastemal, and quantitative RT-PCR analyses were performed (Fig. 6B). Phosphate-buffered saline (PBS)-soaked beads were used as a control. The transplantation of these beads was performed 11 days after limb amputation, and the blastema were harvested after three days after transplantation. *Hoxa13* and *ptch1* expression levels were upregulated by BMP7, FGFs, SHH, BFF, and BFFS (Fig. 6B). Although *lmx1b* expression levels were upregulated by BMP7, BFF, and BFFS, FGFs and SHH were not (Fig. 6B). *Pcna* expression levels were not significantly changed by BMP7, FGFs, and BFF-soaked beads (Fig. 6B). BMP7- and BFF- treated blastema showed particularly strong upregulation of all pattern-related gene expression levels, and BFFS upregulated all gene expression levels compared to the control blastema expression. These results indicate that nerve factor candidates have the potential to regulate patterning-related genes.

Lastly, we investigated whether candidate nerve factors could change morphology (Fig. 7). To test this, pCS2-expression vector plasmids were electroporated
into a regenerating blastema (see the Materials and Methods section). As a control, pCS2-AcGFP vector was electroporated to the blastema and the expression of AcGFP was strongly observed in the blastema (Suppl. fig. 3). The pCS2-AcGFP plasmids resulted in a normal spike (Fig. 7A, D, Table 3). pCS2-\textit{bmp7}, pCS2-\textit{fgf2}, and pCS2-\textit{fgf8} plasmids were electroporated simultaneously into a \textit{Xenopus} blastema. The electroporation resulted in multiple pieces of cartilage and a joint-like structure (Fig. 7B, E, Table 3). Nevertheless, the formation of multiple cartilage elements and the induction of a branched structure like that of a hyperinnervated regenerate was not successful (Fig. 2C, F, 7B, E). Even additional overexpression through the electroporation of pCS2-\textit{shh} did not result in a branch in the spike (Fig. 7C, F, Table 3). Finally, the length of the BFF (pCS2-\textit{Bmp7}, pCS2-\textit{fgf2}, and pCS2-\textit{fgf8} plasmids) vectors-electroporated blastema was shorter than that of the AcGFP vector-electroporated one, and while the BFFS (pCS2-\textit{Bmp7}, pCS2-\textit{fgf2}, pCS2-\textit{fgf8}, and pCS2-\textit{shh} plasmids)-electroporated blastema rescued the length of the regenerates (Suppl. fig. 3). These findings demonstrate that the \textit{bmp}, \textit{fgf}, and \textit{shh} genes together are the responsible candidates for cartilaginous pattern formation in \textit{Xenopus} froglet limb regeneration.
Discussion

Nerve dependence on pattern formation and blastema induction in limb regeneration

Nerve function in the patterning phases of limb regeneration had not previously been investigated. It had lacked attention as the primary focus has been on nerve functions in the blastema induction phase. Recently, it was revealed that fgf and bmp genes could substitute for nerve function in blastema induction in urodele amphibians (Makanae et al., 2014; Makanae et al., 2016). Nerve function similar to that in urodeles was also reported in *Xenopus laevis* (Satoh et al., 2015). Other nerve factors have been also described (Ggf, nAG, and neuregulin; Brockes and Kintner, 1986; Kumar et al., 2007; Farkas et al., 2016). Indeed, several of the nerve factors regulating blastema induction have been identified. Nevertheless, the roles of nerves in patterning remain largely unknown. It was reported that denervation does not affect blastema morphogenesis in urodeles (Stocum, 2011). The nerve influence was instead suggested to be involved in the maintenance of blastema cell proliferation (Stocum, 2011). The present study demonstrated that nerves have positive effects on cell proliferation (Fig. 2J–L); this was also shown in a previous study on *Xenopus* limb regeneration (Suzuki et al., 2005). Furthermore, we demonstrated that hyperinnervation resulted in morphological modification in a *Xenopus* blastema. Moreover, gene expression patterns were changed by the hyperinnervation. Our results suggest the existence of nerve roles in blastema patterning in *Xenopus* limb regeneration.

The nerve factors involved in the patterning of a blastema were unknown, although those involved in the blastema induction phase are relatively obvious as mentioned above. *Bmp* and *fgf* genes are expressed in amphibian DRG neurons.
(Makanae et al., 2014, Satoh et al., 2015, Fig. 5A). It is likely that they are delivered to the limb to regulate the limb regeneration process (Satoh et al., 2016). It was previously reported that FGF and BMP application could induce a blastema in a *Xenopus* froglet limb (Satoh et al., 2015). FGF2, FGF8, and BMP7 applications to wounded *Xenopus* skin resulted in blastema formation (Satoh et al., 2015). The same combination of genes would not, however, be sufficient to induce the same morphological change as hyperinnervation (Fig. 7B, E). The electroporation of *fgf2, fgf8*, and *bmp7* could not induce external morphological changes although multiple cartilage changes were induced (Fig. 7B, E). This suggests the existence of one or more additional factors in the patterning phase of *Xenopus* froglet limb regeneration. *Shh* was examined in the present study as another candidate nerve factor (Fig. 7C, F). *Shh* signaling was previously reported to play a role in *Xenopus* limb blastema patterning (Endo et al., 2000; Yakushiji et al., 2007; Yakushiji et al., 2009). The detection of *shh* in *Xenopus* DRG neurons suggests that *shh* is a potential candidate (Fig. 5A). Yet additional *shh* electroporation to *fgf* and *bmp* genes did not lead to an external morphological change like that seen in a hyperinnervated limb (Figs. 2C, F, 7C, F). Given that this electroporation resulted in multiple cartilage formation in the regenerates, *fgf*, *bmp*, and *shh* genes likely play a role in the patterning process as nerve factors. The detailed identification of the nerve factors involved in the patterning phases requires further investigation.

**Activation of positional information and proliferation in hyperinnervated blastemas**

The hyperinnervated regeneration mainly affected the distal regions of blastemas (Fig. 2). The regenerates formed branch structures at the distal parts of the regenerates, and
separated cartilage was observed in the distal parts of regenerates (Fig. 2E, F). An apical epidermal cap (AEC) plays essential roles in urodele limb regeneration (Stocum, 2011). The AEC maintains blastema cell growth and the de-differentiated state of blastema cells. In *Xenopus* limb regeneration, the AEC is formed by nerves from the newly formed wound epithelium (Endo et al., 2000; Mitogawa et al., 2014). The proportion of proliferated cells is higher in a hyperinnervated blastema than it is in a normal blastema (Fig. 2J–L). Particularly in the distal region of the blastema, the number of BrdU-positive cells clearly differ (Fig. 2J–L). *Hoxa13* expression level was upregulated whereas that of *hoxa11* was not (Fig. 3G, H). *Hoxa13* expression was strongly downregulated by Dorsomorphin in a hyperinnervated blastema and upregulated by BMP7, BFF, and BFFS beads in a normal blastema (Fig. 6). This suggests that the distal portion of a blastema is more sensitive to nerve effects and BMP7. Since AEC is located in the distal part of a blastema, the nerve may influence the AEC to regulate cell proliferation and gene expression. Also, *lmx1b* and *ptch1* expression were up- and down-regulated by BMP (Fig. 6). These results imply that main players among the nervous factors in *Xenopus* froglet limb regeneration are BMP genes. Further investigation is required to confirm this.

The present study demonstrated that hyperinnervation could reactivate *shh* and *lmx1b* expression in a regenerating blastema (Fig. 4). Defects in those gene expressions in a regenerating *Xenopus* blastema have been reported previously (Endo et al., 1997; Matsuda et al., 2001; Endo et al., 2000). In particular, defective *shh* expression in a *Xenopus* blastema has been well studied (Endo et al., 1997; Endo et al., 2000; Yakushiji et al., 2007; Yakushiji et al., 2009). This is because *Shh* knockout in mouse limb development results in a spike-like phenotype in its limb (Sagai et al., 2005). Hh-
signaling inhibition by a chemical component in axolotl limb regeneration resulted in a spike-like phenotype as well (Roy and Gardiner, 2002). The relationship between the shh defect and spike-like morphology may be caused by epigenetic modifications within the shh enhancer domain (Yakushiji et al., 2009). Regeneration-competent stages/animals have unmethylated CpG in the shh enhancer domain (Yakushiji et al., 2009). In contrast, regeneration-incompetent Xenopus frogs have methylated CpG (Yakushiji et al., 2009). Hyperinnervation of a blastema results in shh expression in a regenerating Xenopus froglet blastema (Fig. 4F). Whether nerve factors directly change the epigenetic state or whether proliferation accelerated by the innervation change the epigenetic state remains unknown. Further studies are required for clarification of this point. The shh activation by innervation occurred on the posterior side of the blastema (Fig. 4F). Patched-1 expression and gli3 upregulation were previously confirmed in an Hh-agonist-treated froglet blastema (Yakushiji et al., 2009). These findings suggest that shh signaling is properly transduced in blastema cells. The phenotype of the shh-activated blastema by hyperinnervation was branched. This branching phenotype could not, however, be obtained by shh overexpression (Fig. 7C, Yakushiji et al., 2009). This strongly suggests that the nerve factors responsible for blastema branching are multiple and complicated. Given that an overdose of nerve factors caused by hyperinnervation could reactivate shh, lmx1b, and hoxa13 in a relatively proper domain in a regenerating blastema, a shortage of nerve factors is very likely to be a major cause of the pattern-deficient phenotype. Identification of the nerve factors involved in pattern-forming stages is expected to be an important component in making regeneration-incompetent animals regenerative.
A recent urodele amphibian study suggests a molecular basis for the anterior posterior positional value of skin in limb regeneration (Nacu et al., 2016). This study suggests that, in axolotls, FGF8 is expressed only in the anterior mesenchyme, and that the maintenance of its expression depends on sonic hedgehog (SHH) signaling from posterior tissue (Nacu et al., 2016). In *Xenopus* limb regeneration, *fgf8* is expressed in those blastema mesenchymal cells whereas *shh* is not (Suzuki et al., 2005; Yokoyama et al., 2001; Yokoyama et al., 2011). The phenotype of branch cartilage that emerges after HH-agonist treatment and pCS-xshh expression vector electroporation may depend on non-proper anterior specific *shh* localization; in the hyperinnervated blastema, *shh* expressed in the anterior blastemal mesenchyme results in a branched structure (Yakushiji et al., 2009; Fig. 7). These results suggest the possibility that the finger-like formations seen after hyperinnervation are controlled by a positional information mechanism similar to that which rescues properly localized *shh* expression by hyperinnervation in axolotls. Further investigation of this possibility is needed.
**Tables**

**Table 1. Regeneration of frog *Xenopus laevis* forelimb with hyperinnervation**

<table>
<thead>
<tr>
<th></th>
<th>Spike</th>
<th>Multiple cartilaginous structure</th>
<th>Branched structure</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperinnervation</td>
<td>6</td>
<td>4*</td>
<td>4*</td>
<td>14</td>
</tr>
<tr>
<td>Intact limb</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>with an amputated</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>sciatic nerve</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05; (values significantly different from the corresponding control using the $\chi^2$ test)

**Table 2. The number of shh and lmx1b expression blastemas**

<table>
<thead>
<tr>
<th>Shh</th>
<th>Positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperinnervation</td>
<td>6*</td>
<td>8</td>
</tr>
<tr>
<td>Control (Intact limb)</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>lmx1b</th>
<th>Positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperinnervation</td>
<td>5*</td>
<td>8</td>
</tr>
<tr>
<td>Control (Intact limb)</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

*P<0.05; (values significantly different from the corresponding control using the Fisher’s exact test with Yates’ correction)
### Table 3. Electroporation of the expression vectors

<table>
<thead>
<tr>
<th></th>
<th>Spike</th>
<th>Branched cartilage</th>
<th>Joint-like structure</th>
<th>Branched cartilage with joint-like structure</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bmp7+Fgf2+Fgf8</strong></td>
<td>6(46%)</td>
<td>2(15%)</td>
<td>4(31%)</td>
<td>1(8%)</td>
<td>13</td>
</tr>
<tr>
<td><strong>Bmp7+Fgf2+Fgf8+Shh</strong></td>
<td>1*(13%)</td>
<td>3(38%)</td>
<td>2(25%)</td>
<td>2(25%)</td>
<td>8</td>
</tr>
<tr>
<td><strong>AcGFP</strong></td>
<td>7*(88%)</td>
<td>1(13%)</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

*P<0.05; (values significantly different from the corresponding control using the $\chi^2$ test)
Materials and methods

Animals and surgery

*Xenopus laevis* froglets of 2 to 3 cm nose-to-tail length were housed in aerated water at 22°C. Amputation was performed using forceps and scissors. Hyperinnervation surgery was performed as described previously (Kurabuchi, 1992). Limb amputation at the mid-zeugopod level was performed two weeks after nerve reroute surgery. *Xenopus laevis* froglets were obtained from a private breeder.

Ethical treatment

All protocols and procedures conformed to the policy on the care and use of laboratory animals of Okayama University. All surgery was performed under ethyl 3-aminobenzoate methanesulfonate salt (MS222) anesthesia, and all efforts were made to minimize suffering.

Histological analysis

Histological analysis was performed as previously described (Mitogawa et al., 2015). Dehydrated tissue sections were immersed in tap water to remove optimum cutting temperature (OCT) compound (Sakura Finetek), stained with Alcian blue solution (Wako) for 3 min, washed with water, stained with hematoxylin (Wako) for 5 min, washed with tap water for several minutes, stained with eosin (Wako) solution for 5 min, and finally washed with 70% ethanol. Sections were then dehydrated with ethanol and mounted using Softmount (Wako).

Immunofluorescence and RNA in situ hybridization
Immunohistochemistry of tissue sections was performed as previously described (Satoh et al. 2007) using anti-acetylated alpha-tubulin (Sigma-Aldrich Cat# T7451, RRID: AB_609894, 1/200), anti-BrdU (DSHB Cat# G3G4, RRID: AB_2618097, 1/200), and anti-mouse IgG-Alexa 488 (Thermo Fisher Scientific Cat# A-11017, RRID: AB_2534084, 1/500). Nuclei were counterstained with Hoechst 33258 (Dojindo), and images were captured using an Olympus BX51 microscope. RNA *in situ* hybridization was performed as described previously (Mitogawa et al., 2015). First-strand cDNA was synthesized from total RNA of a stage 52 limb bud. Anti-Digoxigenin Fab antibody fragments (Roche Cat# 11093274910, RRID: AB_514497) were used. Digoxigenin (DIG)-labeled antisense RNA probes for *Xenopus hoxa13*, *lmx1b*, and *shh* were used to perform *in situ* hybridization. The following primers were used for cloning:

*Xenopus hoxa13* FW: ATACTTTGGCAGCGGATATTATCC  
*Xenopus hoxa13* RV: GCTGTCTGACTGATGCACGACATCC  
*Xenopus lmx1b* FW: CCACCTTAGGAGTGCTGCTC  
*Xenopus lmx1b* RV: TGGCATTTGTGGAGGTGTAA  
*Xenopus shh* FW: ATGCTGTTGCGACTCAATCTCTGCTG  
*Xenopus shh* RV: TCAACTGGATTTCGTTGCCATGCCCAGTGG  
*Xenopus bmp7* FW: GCCCCTATGTATTATGCTGGA  
*Xenopus bmp7* RV: TCTTGGCTCTTGGTGCTTT  
*Xenopus fgf2* FW: ATGCGCGCAGGAGCATCACAATCT  
*Xenopus fgf2* RV: TCAGCCTTTTGCGGACATTGGGAGA  
*Xenopus fgf8* FW: GGTCGAAGACAAGGCAACAT  
*Xenopus fgf8* RV: CCGTACATTTCTCCGCTTT
**BrdU labeling**

For the detection of incorporated BrdU, BrdU (100 μg/g bodyweight, Nacalai Tesque) was injected intraperitoneally, and the forelimbs of froglets were fixed 2 h after BrdU injection. Sections were then treated with 2 N HCl for 30 min at room temperature.

**Quantitative RT-PCR and statistical analysis**

RNA preparation for qRT-PCR was performed using an RNeasy Mini Kit with DNase treatment (Qiagen). Samples were prepared from animals that were raised for 11 days after limb amputation and three days after either chemical treatment (Dorsomorphin, 10 μM, Tocris Bioscience; SU5402 10 μM, Calbiochem; Cyclopamine, 5 μM, Nacalai Tesque)- or grafting of protein (mouse BMP7; mouse FGF2; mouse FGF8 and mouse SHH; R&D systems)-soaked beads. Gelatin beads were used as protein sustained-release beads, and were manufactured according to the previously described method (Endo et al., 2015). Air-dried beads were allowed to swell in stock solutions (1 μg/μl). Equal amounts of proteins were used when formulating the combination protein mixture. RT was performed using PrimeScript II (Takara Bio) according to the manufacturer’s protocol. PCR was performed using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems). Analysis was carried out by ABI StepOne™ software v2.1. For statistical analysis of the data, Welch’s t-test or ANOVA with Bonferroni’s correction was used and normalized by ef-1a. The primers were as follows:

*Xenopus hoxa13* FW: ATACTTTGGCAGCGGATATTATCC

*Xenopus hoxa13* RV: GCTGTCTGACTGATGCACGACATCC

*Xenopus hoxa11* FW: CTTCAAGTTCGAGAGACGTG

*Xenopus hoxa11* RV: GTATTGGTATACGGGCACCT
Xenopus *lmx1b* FW: CTCAGCAAAGATCGTCACG

Xenopus *lmx1b* RV: AGGTGACCTCTGAAGATGCC

Xenopus *pitch1* FW: GGACAAGAATCGCAAGCTG

Xenopus *pitch1* RV: GGATGCTCAGGGAACCTTAC

Xenopus *smo* FW: CCTGATGCAACAAGAAAGCA

Xenopus *smo* RV: CAGCTCTTAAGGCAGATGG

Xenopus *pcna* FW: CACCAAACTACACCCCTGT

Xenopus *pcna* RV: GTTGGGTAGGTCAGCCAAG

Xenopus *ef-1a* FW: CAGATTGGTGGCTGGATATGC

Xenopus *ef-1a* RV: ACTGCCTTGATTACTCCTAC

**Cell culture and western blot**

*Xenopus* blastema cells were collected from *Xenopus* limb blastemas two weeks after amputation at the mid-zeugopod level. The samples were minced using a fine knife and were rotated in 0.5% collagenase/70% PBS for 1 h at room temperature. Then, an equal volume of 0.5% trypsin/0.05% EDTA/PBS was added to the solution and rotated for another 30 min. Tissues were dissociated by pipetting and filtered using a cell strainer (20 μm mesh, Falcon). After washing the cells with culture medium for western blot (0.5% FBS/79.5% Dulbecco’s modified eagle medium [DMEM]/20% distilled water/30 μg/ml gentamycin) and for RT-PCR (1% FBS/79% Dulbecco’s modified eagle medium [DMEM]/20% distilled water/30 μg/ml gentamycin), cells were plated on a plastic dish (Falcon, #3001). Cells were incubated at 27°C with 5% CO2. Three days later, proteins were added and incubated for 2h (western blot) or 3 h (RT-PCR). Proteins were used as follows. BMP7, FGF2, and FGF8 were obtained from R&D Systems, and a
concentration of 1 μg/ml was prepared for each protein. The culture medium was refreshed every day. Whole cell lysates were obtained by directly adding sample buffer to cultured cells. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (GE Healthcare). After blocking, the membranes were incubated with anti-phospho Smad1/5/8 (Cell Signaling Technology Cat# 9511, RRID: AB_331671; diluted 1:1000), anti-phospho ERK1/2 (Cell Signaling Technology Cat# 9101, RRID: AB_331646; 1:1000), and anti-α-tubulin (Sigma-Aldrich Cat# T6199, RRID: AB_477583; 1:5000). Blots were washed six times in Tris-buffered saline containing 0.1% Tween 20 and then incubated with secondary antibody. Signals on membranes were visualized with ECL detection reagents (GE Healthcare), and images were captured using LAS 4000 (GE Healthcare).

**Electroporation**

*Xenopus laevis* were anesthetized by MS222 (Sigma) for approximately 10 min. Plasmid DNA solution (each 1 μg/μl) was injected into a blastema using a fine glass capillary. To increase visualization of the injection, Fast Green dye was added to the solution. Immediately after injection, electric pulses were applied (20 V, 50 ms pulse length, 950 ms interval, 10 times) by NEPA21 typeII.
Figure 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.
Figure 2. Cartilaginous structures of hyperinnervation regenerates

(A–C) Bright-field images. (D–F) Skeletal pattern was visualized by Alcian blue and Alizarin red staining. (A, D) A control spike. (B, C, E, F) Hyperinnervated regenerates. (B, E) A multiple cartilaginous structure in a regenerate that was not branched. (C, F) A branched structure. (G, H) Histological analysis by haematoxylin and eosin staining with Alcian blue. (G) A control spike. (H) A hyperinnervated regenerate. (I) The average ratio of the length between controls and hyperinnervated regenerates. * $p = 0.017$ (Welch’s $t$ test, $n = 5$). (J, K) BrdU analysis. BrdU-positive cells two weeks after amputation; blastemas were visualized by immunofluorescence. (L) BrdU-positive cell rates were calculated by BrdU-positive cells/ Hoechst-positive cells. *$p = 0.047$ (Welch’s $t$ test, $n = 3$). Bars represent standard deviation. Control: normal blastema, Hyper: Hyperinnervated blastema. Scale bar in A is 2 mm. Scale bars in G and K are 500 $\mu$m. A–F, G and H, and J and K are the same magnification, respectively. White lines indicate amputation planes.
Figure 3. Proximal-distal-related gene expressions in a hyperinnervated blastema
(A–F) Hoxa13 expressions were visualized by in situ hybridization. (A, D) A stage (St.) 52 limb bud. (B, E) An intact blastema two weeks after amputation. (C, F) A hyperinnervated blastema two weeks after amputation. D, E, and F are higher magnifications of A, B, and C, respectively. (G) Relative expression levels of hoxa13. A control blastema and a hyperinnervated blastema were each separated into two parts, a proximal half and a distal half, and quantitative RT PCR analysis was performed. **p <
0.01 (Multiple comparisons were performed by Bonferroni’s correction). (H) Relative expression levels of *hoxa11*. Bars represent standard deviation of three technically repeat experiments. Scale bars in A and B are 200μm and 500μm, respectively. B and C are the same magnification. Black arrowheads indicate amputation planes.
Figure 4. Dorsal-ventral- and anterior-posterior-related gene expressions in a hyperinnervated blastema

(A–C) Lmx1b expressions were visualized by in situ hybridization. (A) A stage (st.) 52 limb bud. (B) A control blastema two weeks after amputation. (C) A hyperinnervated blastema two weeks after amputation. C’ is a higher magnification of C. (D–F) Shh expressions were visualized by in situ hybridization. (D) A stage (st.) 52 limb bud. (E) A control blastema two weeks after amputation. (F) A hyperinnervated blastema two weeks after amputation. F’ is a higher magnification of F. D: dorsal, V: ventral, A: anterior, P: posterior. Scale bars in A, B, and D are 200 μm, 500 μm and 50 μm, respectively. B, C, E, and F are the same magnification.
Figure 5. Blastema cell proliferation and effects of growth factor

(A) *Bmp7*, *fgf2*, *fgf8*, and *shh* expression in *Xenopus* DRG were visualized by *in situ* hybridization. (B) Phosphorylated Smad1/5/8 (pSMAD) and phosphorylated ERK (pERK) were detected by western blot analysis. α-tubulin is an internal control. (C) A quantitative PCR analysis of proliferating cell nuclear antigen (pcna). Statistical significance was determined via ANOVA followed by Bonferroni's multiple comparison test. (**), significantly different from each group. Bars represent standard deviation of three technically repeat experiments.
Figure 6. Quantitative analyses of Bmp, Fgf, and Shh signalling effects on blastema gene expression

(A) Effects of Bmp, Fgf, and Shh signaling inhibitors on a hyperinnervated blastema. Samples were prepared from animals that were raised for 11 days after amputation of hyperinnervated limbs and three days after chemical treatments or denervation. A denervated blastema was the control in this experiment. Ctrl: Control, Dor: Dorsomorphin, SU: SU5402, Cycl: Cyclopamine, and Den: Denervation. Statistical significance was determined via ANOVA followed by Bonferroni’s multiple comparison test. (**) significantly different from control. Bars represent standard deviation of four technically repeat experiments. (B) Effects of BMP7, FGF2+FGF8, SHH, BMP+FGFs, and BMP+FGFs+SHH protein-soaked bead transplant on blastema gene expression. Samples were prepared from animals that were raised for 11 days after limb amputation and three days after bead transplants. Controls were transplanted with PBS-soaked beads. FGFs: FGF2+FGF8, BFF: BMP7+FGF2+FGF8 and BFFS: BMP7+FGF2+FGF8+SHH. Statistical significance was determined via ANOVA.
followed by Bonferroni’s multiple comparison test. (**), significantly different from control. B7, Fs, SH and BFF indicate significantly different from BMP7-, FGFs-, SHH- and BFF-treated group, respectively. Bars represent standard deviation of four technically repeat experiments.
Figure 7. The $bmp7^{+}fgf2^{+}fgf8^{+}$ and $bmp7^{+}fgf2^{+}fgf8^{+}shh$ electroporated phenotypes (A–C) Bright-field images. (D–F) Alcian blue and Alizarin red staining images. (A, C) An AcGFP electroporated regenerate. (B, E) $Bmp7^{+}fgf2^{+}fgf8^{+}$ electroporated regenerates. (C, F) $Bmp7^{+}fgf2^{+}fgf8^{+}shh$ electroporated regenerates. Scale bar is 2 mm. Black arrow heads in (E) and (F) indicate multiple cartilage structure.
Supplemental figure 1. Quantitative RT PCR analyses of shh signaling genes in hyperinnervated blastema

Relative expression of *ptch1* and *smo* in normal blastema and hyperinnervated blastema two weeks after amputation. *p* < 0.01 (Welch’s *t* test). Bars represent standard deviation of three technically repeat experiments.
Supplemental figure 2. RNA *in situ* hybridization of *Shh* in *Xenopus* DRG

Anti-sense and sense *in situ* hybridization of *shh* in sectioned *Xenopus* DRG. *Shh* were expressed in DRG cell bodies. Scale bar is 200 μm.
Supplemental figure 3. Electroporation of gene expression vector on *Xenopus* limb blastema

(A–C) The *Xenopus* limb blastema three days after pCS2-AcGFP vector electroporation and seven days after amputation. (D–F) Sections of pCS2-AcGFP electroporated blastema. AcGFP-positive cells were observed in the blastema mesenchyme. Scale bars in A and D are 1 mm and 200 μm, respectively. White lines indicate amputation plane. White arrow heads indicate AcGFP-positive cells. (G) The length of the regenerates. GFP: pCS2-AcGFP vector electroporated regenerates, BFF: pCS2-*bmp7*, and pCS2-*fgf2*, and pCS2-*fgf8* vectors electroporated regenerates, BFFS: pCS2-*bmp7*, and pCS2-*fgf2*, pCS2-*fgf8* and pCS2-*shh* vectors electroporated regenerates. Bars represent standard deviation of eight regenerates.
Supplemental movie 1. The feeding and swimming behavior of three-limb-amputated *Xenopus* frogs

*Xenopus* frogs at two weeks after limb amputation with hyperinnervation. Both forelimbs and the left hind limb were amputated on each frog. They were able to feed on bloodworms, swim, and come up for air.

References


Nacu, E., Gromberg, E., Oliveira, C. R., Drechsel, D., Tanaka, E. M., 2016. FGF8 and
SHH substitute for anterior–posterior tissue interactions to induce limb regeneration.

Nieuwkoop, P.D., Faber, J., 1956. Normal table of Xenopus laevis (Daudin). North-
Holland Publ. Comp. Amsterdam.

hoxa11 and hoxa13 expression during patternless limb regeneration in Xenopus. Dev

Induction of the LIM homeobox gene Lmx1 by WNT7a establishes dorsoventral pattern


long-range cis-regulatory module causes complete loss of limb-specific Shh expression
and truncation of the mouse limb. Development 132, 797-803.


Satoh, A., Makanae, A., Nishimoto, Y., Mitogawa, K., 2016. FGF and BMP derived
from dorsal root ganglia regulate blastema induction in limb regeneration in

Satoh, A., Mitogawa, K., Makanae, A., 2015. Regeneration inducers in limb


Acknowledgements

This work was supported by JSPS KAKENHI Grant Numbers JP15J07688 (to KM), JP15K14560 (to AS), and JP26711015 (to AS).
List of publications


