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Evolution of Developmental Control Mechanisms

FGF and BMP derived from dorsal root ganglia regulate blastema induction in limb regeneration in *Ambystoma mexicanum*

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ABSTRACT

Urodele amphibians have a remarkable organ regeneration ability that is regulated by neural inputs. The identification of these neural inputs has been a challenge. Recently, Fibroblast growth factor (Fgf) and Bone morphogenic protein (Bmp) were shown to substitute for nerve functions in limb and tail regeneration in urodele amphibians. However, direct evidence of Fgf and Bmp being secreted from nerve endings and regulating regeneration has not yet been shown. Thus, it remained uncertain whether they were the nerve factors responsible for successful limb regeneration. To gather experimental evidence, the technical difficulties involved in the usage of axolotls had to be overcome. We achieved this by modifying the electroporation method. When Fgf8-AcGFP or Bmp7-AcGFP was electroporated into the axolotl dorsal root ganglia (DRG), GFP signals were detectable in the regenerating limb region. This suggested that Fgf8 and Bmp7 synthesized in neural cells in the DRG were delivered to the limbs through the long axons. Further knockdown experiments with double-stranded RNA interference resulted in impaired limb regeneration ability. These results strongly suggest that Fgf and Bmp are the major neural inputs that control the organ regeneration ability.

1. Introduction

Urodele amphibians have remarkable regeneration ability, and limb regeneration has been studied as representative of it. When a limb is amputated, a regeneration-specific structure, called a regeneration blastema, is formed on the amputation surface (Goss, 1969). Regeneration-incompetent animals cannot grow a regeneration blastema after amputation. Therefore, the blastema induction mechanism is an important landmark to make regeneration-incompetent animals competent. Nerves have been identified as the essential regulator in blastema induction (Goss, 1969; Makanae and Satoh, 2012; Todd, 1823). The absence of nerves in a limb results in the compete inhibition of limb regeneration. Some studies indicate that trophic factors are secreted from nerves to induce a blastema (Brockes, 1984; Nye et al., 2003; Satoh et al., 2012b). Given the importance of the nerve factors, identifying the molecules secreted from the nerves in limb regeneration is the key to understanding this remarkable regeneration ability.

The identification of the nerve factors responsible for successful limb regeneration has been a longstanding challenge. Substance P, glial growth factor, anterior gradient, bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs) have been reported as the neural factors (Brockes et al., 1986; Brockes and Kintner, 1986; Dungan et al., 2002; Globus, 1988; Kumar et al., 2007; Mitogawa et al., 2014; Mullen et al., 1996; Nye et al., 2003; Satoh et al., 2008; Satoh et al., 2015). Although these basically fulfilled the criteria for being nerve factors in limb regeneration (Brockes, 1984), there was no direct evidence that they were secreted from the nerves to the target sites to regulate blastema induction.

The accessory limb model (ALM) is an alternative experimental design of amphibian limb regeneration study that is suitable for investigating the nerve factors in limb regeneration (Endo et al., 2004). The ALM does not involve amputation; instead, a piece of skin is dissected out. The exposed inner tissues are immediately covered with migrating epithelium called wound epithelium within a day. Then, dermal fibroblasts accumulate underneath the wound epithelium and start reconstituting the dermal collagen matrix. Characteristically, urodele amphibians heal skin wounds without scarring (Seifert et al., 2012). In these processes, there is no sign of blastema formation. If nerves are deviated to a wound site, blastema formation is triggered instead of skin wound healing (Endo et al., 2004). The nerves then start interacting with the overlying wound epithelium, which is thought to create a regenerative environment (Satoh et al., 2008). The created regeneration field represses dermal collagen reconstitution and transforms the wound healing process into the regeneration

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process (Satoh et al., 2012b). Blastema cells are induced under such circumstances. Blastema cells are assumed to contain multipotent cells (Hirata et al., 2010; Kragl et al., 2009; Muneoka et al., 1986). Hence, nerve deviation to a wound site leads to the induction of multipotent cells in adults. The ALM provides an ideal opportunity to study the early phase of limb regeneration as it enables a focus on two targets, the skin and the nerves. This simplified experimental design can aid in the identification of nerve factors (Satoh et al., 2015).

The importance of Fgf and Bmp signaling in limb regeneration is highlighted by the ALM (Makanae et al., 2013; Makanae et al., 2014, 2016; Satoh et al., 2011). The application of recombinant Bmp7, Fgf2, and Fgf8 (B7FF) to skin wounds resulted in blastema formation. In other words, B7FF can substitute for the nerve role in blastema induction (Makanae et al., 2014). Fgf2 and Fgf8 application can induce a blastema but cannot induce a limb. Bmp7 application can induce a blastema-like structure but the induced structure does not express blastema marker *Prrx1* (Makanae et al., 2014). Consistently, a Bmp7-induced structure does not result in a limb. Cooperative inputs of Bmp and Fgf signaling are important for successful limb regeneration (Makanae et al., 2014). Recently, it was demonstrated that the same cooperative inputs of Bmp and Fgf signaling could induce regeneration responses of the tail in urodele amphibians and could induce a blastema in *Xenopus laevis* (Makanae et al., 2016; Satoh et al., 2015). Thus, cooperative Bmp and Fgf inputs may be regeneration inducers in broader species and organs.

To determine the nerve factors involved in limb regeneration, additional direct evidence is required. We developed the electroporation method into an axolotl dorsal root ganglia (DRG) in order to investigate DRG-expressing Fgf8 and Bmp7 were transferred along long axons to the ends of axons. Fgf8-AcGFP and Bmp7-GFP plasmid constructs were introduced into axolotl DRGs and signal of green fluorescent protein (GFP) was investigated in a regenerating ALM blastema. Furthermore, Fgf8 and Bmp7 gene expressions in axolotl DRGs were knocked down by the introduction of double-stranded RNA (dsRNA). RNA interference in axolotl DRGs impaired blastema induction. Our results strongly suggest that Fgf and Bmp are the major neural inputs that control the organ regeneration ability.

2. Materials and methods

2.1. Animals and surgery

Axolotls (*Ambystoma mexicanum*) of 8–12 cm nose-to-tail length were housed in aerated water at 22 °C. Their hind limbs, which had never previously been subjected to surgery, were used. Transgenic axolotls were obtained from the Ambystoma Genetic Stock Center (http://www.ambystoma.org/genetic-stock-center). Amputation was performed by forceps and scissors. ALM surgery was performed as described previously (Endo et al., 2004). Housing condition was the same as axolotls' condition. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Okayama University. All surgeries were performed under MS222 anesthesia, and all efforts were made to minimize suffering.

2.2. Plasmid vectors and double-stranded RNAs (dsRNAs)

pCS2-AcGFP was used for the control experiments. We designed Fgf8-AcGFP vector as reported (Yu et al., 2009). Fgf8SS(secretory signal)-AcGFP-$(Gly_4Ser)_3$ linker-Fgf8 fragment synthesized by overlap extension polymerase chain reaction (OE-PCR) with KOD DNA polymerase (Toyobo, Tokyo) was recloned into pAcGFP-C1 vector (Clontech, CA) at *Age*I - *Bam*HI site. The following primers were used in the OE-PCR: Fgf8SS forward primer; CCCACCGGTATGAACAACGAGAGCTCGTCAGTCGT, reverse primer; GCCCTTGCTCACCATTGGGGACTGAACAGTTACCT, AcGFP forward primer;
ACTGTTCAGTCCCCAATGGTGAGCAAGGGCGCCGA, reverse primer;
CTGAACCGCCTCCACCCTTGTACAGCTCATCCATG, linker forward primer;
TCAGGCGGAGGTGGCAGCGGCGGTGGCGGGTCGCC, reverse primer;
CCGCTGCCACCTCCGCCTGAACCGCCTCCACCCTT, Fgf8 forward primer;
CGGCGGTGGCGGGTCGCCTAATTTTACACAGCATG, reverse primer;
CGGGATCCTATCGCGGTACGGAATGTCGAG. pBmp7-AcGFP was made as follows. Axolotl *Bmp7* was amplified by PCR with KOD DNA polymerase from pCS2-axBmp7. In this PCR, the following primers were used: axBmp7 forward primer;
CCCAGATCTATGGATGTGCCTTGGGGGAAC, reverse primer;
CCCGTCGACCCGTGGCAGCCGCATGCCCGC. The PCR fragments of axBmp7 were recloned into pAcGFP-N1 vector (Clontech, CA) at BglII - SalI site. Synthesized plasmids were amplified and purified by an ordinal $CsCl_2$ isolation procedure. Highly concentrated plasmids (3 μg/μl) were used for the electroporation. Axolotl *Pea3* (*etv4*) was isolated by RT-PCR using the following primers; *Pea3* forward, TGAGGGCTTTTACCGATGAC; *Pea3* reverse, TTGCTGTAGTGCTGCGTAGG. Axolotl *Pea3* was in pTAC2 vector. ptdTomato-N1 vector was purchased from Clontech (CA).

dsRNA-*AcGfp*, dsRNA-*Fgf8* and dsRNA-*Bmp7* were synthesized as follows. PCR fragments were prepared from pTAC2-AcGFP, pTAC2-*axFgf8* and pTAC2-*axBmp7* by KOD polymerase with the following primers: M13 forward primer; GTAAAACGACGGCCAGTT, reverse primer; ATCATCTAATACGACTCACTATAGGGCAGGAAACAGCTATGAC. The PCR products have T7 RNA polymerase binding sequences in both ends. RNA was synthesized by T7 RNA polymerase (Takara Bio, Japan). The synthesized RNAs were purified by chloroform extraction and ethanol precipitation. A high concentration (3 μg/μl) of dsRNA was prepared and used for the electroporation.

2.3. Electroporation

Axolotls were anesthetized by MS222 (Sigma, St. Louis) for about 10 min (depending on the animal size) and placed on ice for 1 h in order to slow their heartbeat. Icing animals contributes greatly to good surgical recovery. The animals were laid out and the upper-lateral region above a hind limb was opened using fine scissors (Fig. 1A). To expose the DRGs existing under diapophysis, bones were cut out using scissors. Attached soft tissues were cleaned out with forceps and DNA or dsRNA solution was injected using a fine glass capillary. To increase the visual of the injection, Fast green dye was added to the solution. Immediately after injection, electric pulses were applied (15 V, 50 ms pulse length, 950 ms interval, 10 times). DRGs in the contralateral side were used for the control experiment (mock electroporation). Wounds were closed and wiped well. The animals were then placed on ice for at least 4 h. During the cooling, the hind limbs were amputated. For the first 3 post-operative days, special attention must be paid to water cleanliness to avoid infection.

2.4. Sectioning and histological staining

Samples were fixed with 4% paraformaldehyde for 1 d at room temperature. If necessary, decalcification by 10% EDTA was performed for 1 d. Samples were embedded in O. C. T. compound (Sakura finetek, Tokyo) following 30% sucrose/phosphate-buffered saline (PBS) treatment for approximately 12 h. Frozen sections of 14 μm thickness were prepared using Leica CM1850. The sections