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Cooperative inputs of Bmp and Fgf signaling induce tail regeneration in urodele amphibians

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ABSTRACT

Urodele amphibians have remarkable organ regeneration ability. They can regenerate not only limbs but also a tail throughout their life. It has been demonstrated that the regeneration of some organs are governed by the presence of neural tissues. For instance, limb regeneration cannot be induced without nerves. Thus, identifying the nerve factors has been the primary focus in amphibian organ regeneration research. Recently, substitute molecules for nerves in limb regeneration, Bmp and Fgfs, were identified. Cooperative inputs of Bmp and Fgfs can induce limb regeneration in the absence of nerves. In the present study, we investigated whether similar or same regeneration mechanisms control another neural tissue governed organ regeneration, i.e., tail regeneration, in *Ambystoma mexicanum*. Neural tissues in a tail, which is the spinal cord, could transform wound healing responses into organ regeneration responses, similar to nerves in limb regeneration. Furthermore, the identified regeneration inducer Fgf2+Fgf8+Bmp7 showed similar inductive effects. However, further analysis revealed that the blastema cells induced by Fgf2+Fgf8+Bmp7 could participate in the regeneration of several tissues, but could not organize a patterned tail. Regeneration inductive ability of Fgf2+Fgf8+Bmp7 was confirmed in another urodele, *Pleurodeles waltl*. These results suggest that the organ regeneration ability in urodele amphibians is controlled by a common mechanism.

1. Introduction

Urodele amphibians have a remarkable ability to regenerate complex organs/tissues, such as limbs. In contrast, most amniotes do not have the organ regeneration ability in most parts. When the regenerative animals start regeneration, a typical structure called a regeneration blastema forms at the damaged place. Non-regenerative animals or organs fail to form the blastema. Hence, revealing mechanisms that induce blastemas in regenerative animals is important and understanding the detailed mechanisms of organ regeneration would help to develop a new clinical treatment in amniotes.

Our understanding of organ regeneration has advanced by studying limb regeneration. A regeneration blastema is induced after limb amputation in urodele amphibians (Goss, 1969). A regeneration blastema consists of undifferentiated stem-like cells termed blastema cells. Some blastema cells are unipotent or multipotent (Hirata et al., 2010; Kragl et al., 2009; Muneoka et al., 1986). Muscle-derived blastema cells usually participate in the muscle tissue regeneration in limbs. On the other hand, dermis-derived blastema cells can participate in the regeneration of several tissue types, such a cartilage and ligament (Hirata et al., 2010; Kragl et al., 2009; Muneoka et al., 1986). The induction of multipotent cells from matured tissues is involved in limb blastema formation. Understanding the mechanisms that underlie blastema induction is important not only because it is the key phenomenon in organ regeneration but also because it involves endogenous reprogramming of differentiated cells into multipotent blastema cells.

Understanding the mechanisms that underlie blastema induction in limb regeneration has been a challenge for a long time. Approximately 200 years ago, it was demonstrated that blastema induction is prevented after a limb is denervated, suggesting the essential nerve roles in blastema induction (Todd, 1823). This nerve-dependent blastema induction in limb regeneration was confirmed in some amphibians (Endo et al., 2000; Kumar and Brockes, 2012). Given the importance of the presence of nerves for regeneration, the nerve factors that could be responsible for blastema induction have been sought. To identify the neural inputs involved in limb regeneration, an alternative experimental system termed the accessory limb model (ALM) was established (Endo et al., 2004; Makanae and Satoh, 2012; Mitogawa et al., 2014; Satoh et al., 2007). According to ALM, three steps are necessary to obtain a limb: 1) skin wounding, 2) nerve deviation, and 3) skin

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grafting from the contralateral limb side of the wounded region. To obtain a blastema, only steps 1 and 2 are required. It is assumed that the skin grafting is necessary to enable sustained growth of an induced ALM blastema, but it is not necessary for blastema induction (Endo et al., 2004). Because ALM does not require limb amputation, it is possible to simplify the study of limb regeneration. Briefly, blastema induction can be studied by focusing on only two targets, skin and nerves. Skin wounding in an axolotl limb results in skin wound healing (Denis et al., 2013; Seifert and Maden, 2014); however, it never results in limb/blastema formation (Endo et al., 2004) nerves are deviated toward the wounded skin, skin wound healing responses are transformed into blastema induction responses. Molecules that can transform wound healing responses into blastema induction responses had been investigated. Recently, it was revealed that cooperative inputs of Fgf- and Bmp-signaling could substitute for the nerve functions in ALM blastema induction (Makanae et al., 2013, 2014; Satoh et al., 2011, 2015). The inductive inputs (Bmp7+Fgf2+Fgf8; B7FF) were confirmed in two urodeles, *Ambystoma mexicanum* and *Pleurodeles waltl*. Moreover, B7FF induced a blastema in a limb of the anuran amphibian *Xenopus laevis* (Mitogawa et al., 2014; Satoh et al., 2015). Hence, the inductive inputs can be expected in a wide range of amphibian species. There is a little room for argument whether B7FF are true nerve factors because it is difficult to delete *Fgf* and/or *Bmp* expression in amphibian nerves. However, B7FF are strong candidates as nerve factors, which are responsible for limb blastema induction.

Do common fundamental mechanisms regulate the induction of organ regeneration in other organs? As mentioned above, axolotls can regenerate various body parts, and it is known that regeneration in some of those body parts is nerve-dependent (Kumar and Brockes, 2012). This implies a common nerve-dependent regulatory mechanism for organ regeneration. The regeneration of a tail can be the first step used to test this idea. Tail regeneration in urodele amphibians has been investigated for a long time. Similar to limb regeneration, the tail of urodeles consists of multiple cell types and forms a complex three-dimensional structure, and tail regeneration is regulated by the neural tissue, which is the spinal cord (SC) (Holtzer et al., 1955; Taniguchi et al., 2008). In the presence of SC, an amputated tail induces a regeneration blastema. Thus, aspects of tail regeneration appear to be quite similar to those of limb regeneration. In the present study, we found that the axolotl SC has the ability to transform wound healing responses to organ regeneration responses as well as nerves in limb regeneration. As observed in limb regeneration, *Fgf* and *Bmp* genes are expressed in the axolotl SC and the application of recombinant Fgf and Bmp proteins could substitute for the regenerative roles of SC, resulting in the induction of a tail-like structure. To confirm these findings, the same procedures were investigated in another species, *P. waltl,* and the same results were observed. These results strongly suggest that similar regulatory mechanisms govern tail and limb regeneration in urodele amphibians and imply the existence of fundamental regeneration mechanisms controlling organ regeneration throughout the body of a regeneration-capable animal.

2. Materials and methods

2.1. Animals and tail blastema grafting

Axolotls (*Ambystoma mexicanum*) with a nose-to-tail length of 8–12 cm were obtained from private breeders and housed in aerated water at 22 °C. Newts (*P. waltl*) with a nose-to-tail length of 5–7 cm were obtained from Takeuchi laboratory, Tottori University, Japan. Newts and axolotls were housed under the same conditions. Green fluorescent protein (GFP) transgenic axolotls were obtained from the Ambystoma Genetic Stock Center (AGSC). For blastema grafting, smaller animals were selected. Lateral tail blastemas were induced using a bead in the GFP transgenics as described below. Simultaneously, the distal tip of the tail of normal animals was clipped. Blastemas in the lateral tail of the transgenic animals and the clipped tail of the normal animals grew 6 days after the surgery. Blastemas of the transgenic animals were removed from the lateral tails using scissors and forceps. Epidermal tissues were removed as much as possible. Then, the blastema mesenchyme was inserted into the tail blastema of the normal animal.

2.2. Inhibitor treatment

SU5402 (Calbiochem) and dorsomorphin dihydrochloride (DMD) (Tocris Bioscience, Bristol, UK) were dissolved in DMSO (Nacalai Tesque, Kyoto, Japan) and DDW, respectively, to prepare a 10 mM stock solution. For the inhibitor treatment in tail regeneration experiments, we kept the animals in the presence of SU5402 (10 μM), DMD (20 μM), or DMSO, in water from the day when the bead was grafted to day 14. Water was refreshed every 2 days. After the treatment, animals were returned to original housing conditions.

2.3. Bead grafting

Gelatin beads were used as protein sustained-release beads and were manufactured following the previously described method (Endo et al., 2015). Air-dried beads were allowed to swell in stock solutions (1 μg/μl) that were prepared according to the manufacturer's instructions. Equal amounts of proteins were used when formulating the combination protein mixture (for example, the Bmp7, Fgf2, and Fgf8 mixture contained 0.33 μg/μl of each protein). Beads were soaked in the protein mixture for at least 3 h on ice. The beads can be stored at 4 °C but should be used within a week, and the stock solution can be stored at −80 °C but it is recommended to be used within a month of opening. Bmp7 (mouse), Fgf2 (mouse), and Fgf8 (human/mouse) were obtained from R&D systems (Minneapolis, USA). For control experiments, the gelatin beads were soaked in phosphate-buffered saline (PBS).

2.4. Accessory tail induction

Accessory tail induction procedures are illustrated in Sup. Fig. 1A and B. First, the lateral wound is created. To manipulate SC, muscles and some related tissues were removed with the tail skin. A skin graft was prepared from the contralateral side of the tail. SC was rerouted to the wound, and the skin graft was placed near the rerouted SC. For the bead grafting, tail wounding and skin grafting were performed without SC rerouting. The bead was implanted 3 days after the wounding.

2.5. Sectioning and histological staining

Tissue samples were fixed with 4% paraformaldehyde for 1 day at room temperature. Decalcification by 10% EDTA was performed for 1 day. Samples were cryoprotected in 30% sucrose/PBS solution for approximately 12 h and then embedded in O.C.T. (Sakura Finetech, Tokyo, Japan). Frozen sections were cut at a thickness of 14 μm using a Leica CM1850 cryostat. The sections were dried using an air dryer, then stored at −80 °C until use.

Standard hematoxylin and eosin (HE) staining was used for histology. To visualize cartilage formation, Alcian blue staining was performed before HE staining. In brief, sections were washed in tap water several times to remove the O.C.T. compound. Then,