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Fgf- and Bmp-signaling regulate gill regeneration in *Ambystoma mexicanum*

Nanami Saito, Koki Nishimura, Aki Makanae, Akira Satoh *

Okayama University, Research Core for Interdisciplinary Sciences (RCIS), 3-1-1, Tsushimanaka, Kitaku, Okayama, 700-8530, Japan

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ABSTRACT

Gill regeneration has not been well studied compared to regeneration of other appendages, such as limb and tail regeneration. Here, we focused on axolotl gill regeneration and found that Fgf- and Bmp-signaling are involved in their gill regeneration mechanism. Axolotls have three pairs of gill rami, and each gill ramus has multiple gill filaments. The gills consist of mesenchyme rich in extracellular matrix and epidermis. The gill nerves are supplied from the trigeminal ganglia located in the head. Denervation resulted in no gill regeneration responses. Nerves and gills express *Bmp* and *Fgf* genes, and treating animals with Fgf- and Bmp-signaling inhibitors results in phenotypes similar to those seen in denervated gills. Inducing an accessory appendage is a standard assay in amphibian regeneration research. In our study, an accessory gill could be induced by lateral wounding, suggesting that thin axon fibers and mesenchymal *Fgf*s and *Bmp*s contributed to the induction of the accessory structure. Such accessory gill induction was inhibited by the denervation. Exogenous Fgf2+Fgf8+Bmp7, which have been determined to function as a regeneration inducer in urodele amphibians, could compensate for the effects denervation has on accessory blastema formation. Our findings suggest that regeneration of appendages in axolotls is regulated by common Fgf- and Bmp-signaling cascades.

1. Introduction

Axolotls have the ability to regenerate many of their organs throughout their life, and many appendages can be restored after amputation (Goss, 1969). The limbs, the tail, and the gills are major appendage organs of axolotls. Because of the appearance characteristics of the appendages, the likelihood of severe damage is high. Axolotls often lose or suffer serious damage to those organs but can usually regenerate them completely (Tsonis, 1996; Wallace, 1981). Regeneration of appendages in salamanders has been well studied.

The gills of the axolotl represent such regenerative organs. Since they undergo a neotenic life cycle, axolotls keep their external gills throughout their life (Rosenkilde and Ussing, 1996). The external gills are thought to play a role in respiration. However, the details of their function are still widely unknown. It has been believed that axolotls use three methods for respiration (Gahlenbeck and Bartels, 1970; Piiper and Scheid, 1975). Axolotls develop lungs/air sacs even though they spend their whole life in water. When supplied with sufficient oxygen in water, they barely use their (not the) lungs. The skin also plays a significant role in their respiration (Walter and Victor, 1963), as the majority of the required oxygen is believed to be absorbed through the skin. Not surprisingly, the gills also serve an important function in respiration. An axolotl possesses three gill ramus pairs. Each gill ramus grows many gill filaments which contain extensive networks of blood capillaries. The proximal region of the gill ramus contains muscle tissues used in gill movement. Multiple filaments increase the surface area, and swinging the gill ramus permits fresh water flow, allowing for a larger volume of gas to be exchanged. When cultured together under laboratory conditions, this feature may cause loss of the external gills due to a reflexive eating action by other axolotls. The animals have the ability to regenerate the lost gills just like other organs, and gill regeneration appears to be a familiar phenomenon. However, research on this topic is scarce.

It has been determined that cooperative inputs of Fgf- and Bmp-signaling serve as organ (appendage) regeneration inducers in amphibians (Makanae et al., 2013; Makanae et al., 2014a, b; Makanae and Satoh, 2012; Satoh et al., 2011; Satoh et al., 2016; Satoh et al., 2015). Nervous tissue is well known as essential for successful limb and tail regeneration (Makanae et al., 2016). Removal of the nerves from a limb leads to absence of regeneration responses (Goss, 1969; Todd, 1823; Tsonis, 1996; Wallace, 1981). Hence, molecules that can substitute for nerve roles in limb regeneration had been sought for a long time. It was shown that Fgf2+Fgf8+Bmp7 (or Bmp2) can meet all criteria for nerve-substitute molecules (Makanae et al., 2014b, 2016; Satoh et al., 2016). In axolotls, *Fgf* and *Bmp* genes are expressed in neural cells in the

* Corresponding author. Tel.: +81 86 251 8421; fax: +81 86 251 8705.
E-mail address: satoha@cc.okayama-u.ac.jp (A. Satoh).

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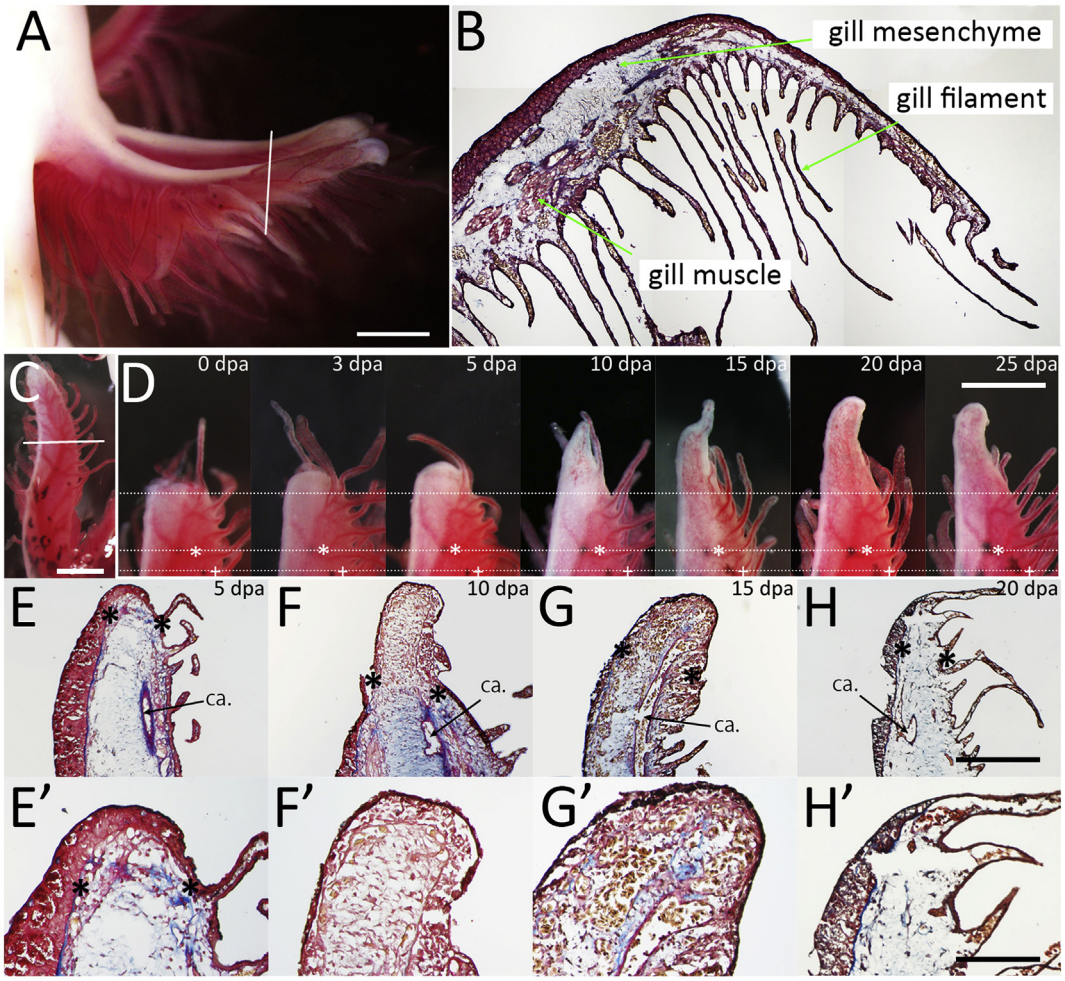

Fig. 1. Regeneration process of the axolotl gill. (A) Intact external gill. (B) Histological observation of the intact gill. Trichrome staining was performed on the section. The dorsal side is up and the ventral side is down. (C, D) Time course of gill regeneration. The line in C indicates the approximate amputation site. The asterisks and the white cross marks in D point out the identical branching of blood vessels. The white dotted lines in D indicate the same level based on the asterisks and the white cross marks. (E–H) Histological observation. Trichrome staining was performed on the sections. Asterisks in E–H indicate the end of collagen layers, pointing out the approximate amputation plane. Scale bars in A, C, D, H, and H′ are 2000, 2000, 2000, 500, and 200 μm, respectively. ca. = gill capillary.

dorsal root ganglia (Makanae et al., 2014a). Fgf2+Fgf8+Bmp7 can induce limb regeneration responses in the absence of nerves in axolotls and newts, suggesting that those molecules can serve as substitutes for nerve functions in limb regeneration. Moreover, the same combination can induce tail regeneration (Makanae et al., 2016). It was shown that the same inputs can induce a blastema in the limbs of *Xenopus laevis* froglets even though their limbs show hypomorphic regeneration ability (Satoh et al., 2015). Thus, the cooperative inputs of Fgf- and Bmp-signaling are likely to have regenerative effects in multiple organs and organisms.

In the present study, we focused on axolotl gill regeneration. Because of the lack of insights in axolotl gills, we first describe histological observations in axolotl gill regeneration. Axolotl gills consist of epidermal and mesenchymal cells. Mesenchymal cells are sparsely distributed, and the majority of the mesenchymal region is composed of extracellular matrix. Extensive capillary networks are also apparent in the gill ramus and filaments. Thin axon fibers can be identified throughout the gill

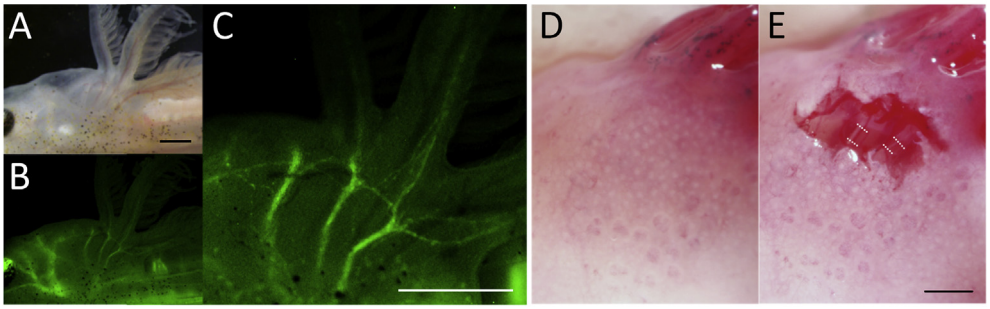

Fig. 2. Nerve routes and denervation procedures in axolotl gills. (A–C) Nerve routes were visualized using green fluorescent protein (GFP), driven by the βIII tubulin enhancer/promoter. To visualize GFP signals through the skin, smaller animals were used. (A) Bright-field view. (B, C) Dark-field view. Three major nerve routes appear to extend toward the gills. (D, E) The two rostral nerves were targeted for dissection. Scale bars are 2 mm.

ramus. Nerve fibers with axons projecting to each gill ramus are bundled in the most-proximal region, which was targeted in our denervation experiment. As expected, gills can be regenerated after amputation, and an intact gill and a regenerated gill cannot be distinguished 1 month after amputation. However, denervation impaired gill regeneration, suggesting a dependence on nervous tissues. Chemical inhibition of Fgf- or Bmp-signaling delayed gill regeneration, suggesting the involvement of Fgf- and Bmp-signaling in gill regeneration as well as limb and tail regeneration. The present study also revealed that axolotl gills and nerves with axons projecting to a gill ramus express *Fgf* and *Bmp* genes in the mesenchyme. Moreover, induction mechanisms of an accessory gill involve Fgf- and Bmp-signaling. Such induction of an accessory structure is consistent with findings in limb and tail regeneration. Our results suggest that gill regeneration is regulated by Fgf- and Bmp-signaling, and that cooperative inputs of Fgf- and Bmp-signaling are conserved and represent the principal mechanism of limb, tail, and gill regeneration, at least in *Ambystoma mexicanum*.

2. Materials and methods

2.1. Animals

Axolotls (*Ambystoma mexicanum*) with a nose-to-tail length of 8–12 cm were used for most experiments. The axolotls were housed in aerated water at 22 °C. Axolotls with a nose-to-tail length of 3–4 cm were used for inhibition experiments. For the observation of neural fibers, βIII tubulin-GFP animals, which were kindly provided from Elly Tanaka Lab, were used. Gills were amputated at one-third from the distal tip. To determine the location for the amputation, a picture was taken first, then the length of the gill was measured using Photoshop CS5 software (Adobe, San Jose, CA, US).

2.2. Bead grafting

Gelatin beads were used to provide sustained-release proteins. The beads were manufactured following a previously described method (Satoh et al., 2011). The air-dried beads were allowed to swell in stock solutions (1 μg/μl) which 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 of each protein per μl. The beads were soaked in the protein mixture for at least 3 h on ice. After immersion, the beads can be stored at 4 °C, but they should be used within a week. Similarly, the stock solution can be stored at −80 °C, but it is recommended that it be used within 1 month after opening. Bmp7 (mouse), Fgf2 (mouse), and Fgf8 (mouse) were obtained from R&D Systems (Minneapolis, MN, US). As controls, gelatin beads were soaked in phosphate-buffered saline (PBS). Before bead grafting, a denervation procedure was carried out, and a piece of gill skin was removed. Beads were applied to the wound in the denervated gill 3 days after wounding. A small slit and a tunnel to the wound area was made using small scissors and sharp forceps. A protein-soaked bead was placed through the slit and the tunnel under the newly formed wound epidermis.

2.3. Inhibitor treatment

SU5402 (Calbiochem, San Diego, CA, USA) and dorsomorphin dihydrochloride (DMD; Tocris Bioscience, Bristol, UK) were dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) and double-distilled water (DDW), respectively, to prepare a 10-mM stock solution. For inhibitor treatment in the gill-regeneration experiments, we kept the animals in the presence of either SU5402 (10 μM), DMD (2 μM), or DMSO, in water. The water was changed every 2 days. After the treatment, the animals were returned to their original housing conditions.

2.4. Accessory gill induction

To induce the formation of an accessory gill, lateral wounding is sufficient. A lateral wound at the dorsoventral border was made at the distal one third of the ramus. A shallow wound was created using forceps and microscissors.

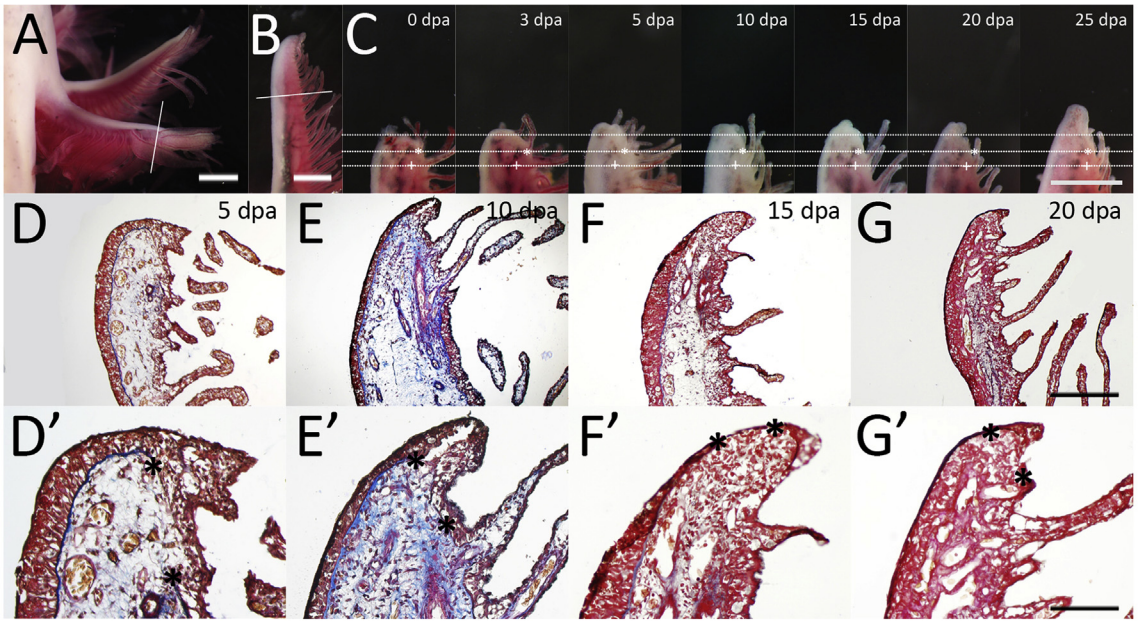

Fig. 3. Denervation in axolotl gill regeneration. (A, B) Intact gills. The white lines indicate the presumptive amputation sites. (C) Regeneration process in the denervated gill. The asterisks and the white cross marks in D point out the identical capillary branching. The white dotted lines in D indicate the same level based on the asterisks and the white cross marks. (D–G) Histological observation. Trichrome staining was performed on the sections. Asterisks in D′–G′ indicate the end of collagen layers, pointing out the approximate amputation plane. Scale bars in A, B, C, F, and F′ are 2000, 2000, 2000, 500, and 200 μm, respectively.

2.5. Sectioning, histological staining, and in situ hybridization

Tissue samples were fixed with 4% paraformaldehyde/PBS for 1 day at room temperature, and the fixed tissues were treated in 30% sucrose/PBS at 4 °C for 1 day. Then, the samples were embedded in O.C.T compound (Sakura Finetech, Tokyo, Japan). Frozen sections of 14-μm thickness were prepared using a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany). The sections were dried under an air dryer. For histological observation, standard trichrome staining was performed using the Trichrome Stain (Masson) Kit (Sigma-Aldrich, St. Louis, MO, USA). The stained sections were mounted using Softmount (Wako Pure Chemical Industries, Osaka, Japan). Immunofluorescence on the sections was carried out according to previously reported methods (Satoh et al., 2007). Acetylated α-tubulin (sc-23950, 1:500; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-mouse IgG Alexa 594 (A21203, 1:500; Invitrogen, Carlsbad, CA, USA) were used for the immunofluorescence procedure. Images were captured using an Olympus BX51 system (Olympus Life Science, Waltham, MA, USA). RNA probes and *in situ* hybridization procedures have previously been described (Makanae et al., 2014a,b).

3. Results

3.1. Gill regeneration in Ambystoma mexicanum

The gill regeneration process in axolotls has not been examined for a long time. Gill regeneration depends largely on the health of the animals. Axolotls easily resorb their gills in bad water conditions, and such resorbed gills under these conditions usually do not regenerate at all. Therefore, we used well-housed animals in the present experiments. Amputation was performed at the distal one-third of the ramus (Fig. 1A). A blastema was visible within 10 days after the amputation (dpa; Fig. 1C and D), and the regenerated gills recovered blood circulation within 15 dpa. Signs of gill filament formation were observable by 20 dpa. To investigate the regeneration process further, sections were made (Fig. 1B, E–H). In the non-amputated region (the stump), extracellular matrix occupied the mesenchymal domain (Fig. 1B). In fact, only a few mesenchymal cells could be identified. The epidermis showed differences between the dorsal gill ramus and the ventral gill filament sides. The dorsal epidermis was thickened while the ventral epidermis was relatively thinner (Fig. 1B). In the proximal region of the gill, there are gill muscles in the ventral side (Fig. 1B). The amputation plane was already closed 5 dpa (Fig. 1E, E′). No apparent cell accumulation was observed at this time. Ten days are usually sufficient to form a blastema (Fig. 1F), as was the case in the present study. The cell population in the blastema appeared much denser compared with the stump region (Fig. 1F'). The blastema grew and tissue differentiation became apparent 15 dpa (Fig. 1G, G′). By this time, the capillaries had penetrated the blastema region (Fig. 1G). By 20 dpa, it became difficult to distinguish between the regenerated region and the stump (Fig. 1H, H'). The gill filaments were well formed, and the epidermis exhibited normal morphology. These findings indicate that most axolotl gill regeneration processes take place within 20 dpa.

3.2. Nerve dependence of gill regeneration in axolotls

Nerve tissues play an important role in appendage regeneration. It is well known that blastema formation in limb regeneration depends on nerve presence (Goss, 1969; Todd, 1823; Tsonis, 1996; Wallace, 1981), and that denervation of a limb results in complete loss of the ability to regenerate. In our present study, we thus investigated nerve dependence in gill regeneration (Figs. 2 and 3). First, to clarify nerve routes in axolotl gills, βIII Tubulin-GFP transgenics, in which all nerve fibers are labeled with GFP, were observed. An axolotl possesses three pairs of gill rami and three major axons extend toward the gill rami (Fig. 2A–C). Denervation was performed by removing sections of the axons of about 1 mm in length, creating a gap between axon routes (Fig. 2D and E). We targeted

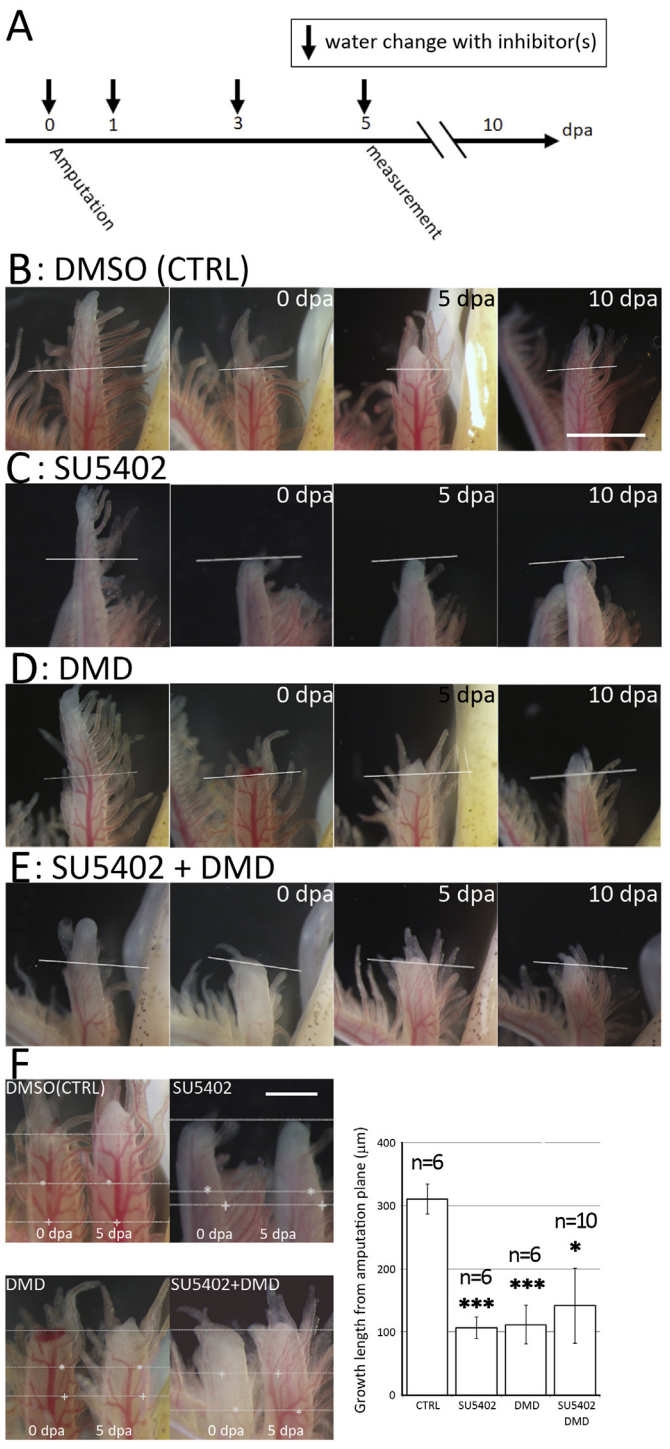

Fig. 4. Fgf- and Bmp-signaling inhibition in gill regeneration. Smaller animals were used for this experiment. (A) Experiment schedule. (B) Dimethyl sulfoxide (DMSO) treatment as positive control. Blastema formation was observable. (C) Gill treated with SU5402, an Fgf-signaling inhibitor. (D) Gill treated with dorsomorphin (DMD), a Bmp-signaling inhibitor. (E) Double-treated gill (SU5402 + DMD). (F) The length of the blastema was measured at 5 days after amputation (dpa). The four right panels in F were manipulated using Photoshop to measure the blastema. Asterisks and cross marks indicate identical positions based on the capillary pattern. The number above the each bar indicates the number of the sample. T-test was used for the statistical analysis. * = $p < 0.05$, *** = $p < 0.01$. B–E show the same magnification. Scale bars in B and F are 2 and 1 mm, respectively.

the axons supplying the two dorsal gills, and regeneration was investigated in the denervated gills (Fig. 3). As expected, regeneration ability in the denervated gills was impaired (Fig. 3A–C). No apparent blastema formation was confirmable in 20 dpa. A small growth from the stump was confirmable at 25 dpa (Fig. 3C). This may have been due to the recovery of the gill nerves. The section revealed no apparent cell accumulation around the amputation plane (Fig. 3D–G). A small extension was usually observable, but these small extensions appeared to be filled with hematocytes (Fig. 3E). This result suggests that nerves play a major role in blastema induction in gill regeneration as well as in limb regeneration.

3.3. Fgf- and Bmp-signaling in axolotl gill regeneration

We previously showed that *Fgf* and *Bmp* genes were expressed in the dorsal root ganglia of axolotls, and their proteins could serve as a substitute for nerve functions in limb regeneration (Makanae et al., 2014a). In fact, nerves in limbs express *Fgf* and *Bmp* genes (Makanae et al., 2014a). Therefore, in the present study, we focused on Fgf- and Bmp-signaling in gill regeneration since gill regeneration appears to be dependent on the presence of nervous tissue. We used chemical compounds to block signaling cascades. The animals were kept in water containing the signal-blocking chemical, and blastema growth was recorded. The experimental schedule is shown in Fig. 4A. SU5402, an Fgf-signaling inhibitor, slowed the pace of regeneration, compared with controls (Fig. 4B, C, F). DMD, a Bmp-signaling inhibitor, also decreased the pace of regeneration (Fig. 4B, D, F). Double treatment led to similar results as did single treatment (Fig. 4B, E, F). These results imply that Fgf- and Bmp-signaling are involved in gill regeneration.

In limb regeneration, nerves are a source of Fgfs and Bmps, and nerves provide those proteins to a wound to induce regeneration. Thus, nerves with axons projecting to gills were investigated to determine *Fgf* and *Bmp* gene expression patterns. Nerve plexuses (trigeminal ganglia) were dissected. Axon fibers supplying the gills were traced to identify the trigeminal ganglia. To investigate gene expression patterns in the neural cells in the trigeminal ganglia, *in situ* hybridization for visualizing gene expression and immunofluorescence (Acetylated α-tubulin) for visualizing neural cells were performed on identical sections. This experiment revealed that *Bmp2*, *Bmp7*, *Fgf2*, and *Fgf8* were expressed in the trigeminal ganglia (Fig. 5A–E). In the trigeminal ganglia, many axon fibers are running inside, where no *in situ* hybridization signal was detected. Most signals could be seen within the cell body of the neural cells (Fig. 5A3–E3, arrows). This finding suggests that nerves from trigeminal ganglia are potential sources of Fgf and Bmp proteins.

3.4. Induction of accessory structures in axolotl gills

To investigate the role of nerves in blastema induction more directly, a nerve deviation method has been used in limb regeneration studies (Endo et al., 2004; Satoh et al., 2007). A lateral skin wound results in simple skin wound healing in a limb. Nerve rerouting after skin wounding results in blastema formation, indicating the essential nerve roles in the blastema induction process. We investigated the similarities of nerve functions in blastema formation in gill and limb regeneration. Unexpectedly, lateral wounding resulted in the formation of a blastema leading to growth of an accessory gill without nerve rerouting (Fig. 6A, C). The blastema was visible 10 days after wounding (Fig. 6A). The induced blastema kept growing and finally formed the accessory gill (Fig. 6C). Such accessory gill induction from a wounded gill could be seen in 8 out of 10 samples (Table 1). The accessory gill showed well-developed gill filaments (Fig. 6C–F), with a morphology and histology quite similar to original gills (Fig. 6D–F). Blood vessels and nerve fibers were also recognizable (Fig. 6D–F). This indicates that the formation of an accessory blastema on a gill can be induced by simple wounding. The formation of such an accessory gill may be explained as follows: 1) Wounding results in cutting many axons running in a gill; 2) Gill cells express *Fgf* and *Bmp* genes. In our study, we first investigated the axon fibers in a gill. Gills are not only a part of the respiration system, but they also serve as sensing organs. Thus, thin sensory axons are running across the gills. The axons were visualized using immunofluorescence (Fig. 6G). When creating a wound in the gill, those axons are inadvertently damaged. Correspondingly, the induced accessory gill contained thin axon fibers (Fig. 6G). Denervation prevented the formation of an accessory gill (Fig. 6B, Table 1). Thus, the axons projecting into a gill played a role in the formation of an accessory gill. We also investigated the gene expression pattern in a gill. It has previously been shown that *Fgf2*, *Fgf8*, *Bmp2*, and *Bmp7* are expressed in the axolotl limb nerves, and that they are responsible for limb regeneration (Makanae et al., 2014a). Therefore, we investigated the expression patterns of the same gene set in axolotl gills (Fig. 7). The axolotl gill was sectioned transversely (Fig. 7A, G). First, the gene expression pattern in the intact gill was investigated using *in situ* hybridization. *Bmp2* and *Fgf2* signals could be detected (Fig. 7C, E). These *Bmp2* and *Fgf2* signals were observed in most tissues except muscles (Fig. 7C, E). *Bmp7* and *Fgf8* could not be observed (Fig. 7D, F). *Col1A2* served as a positive control (Fig. 7B). We also

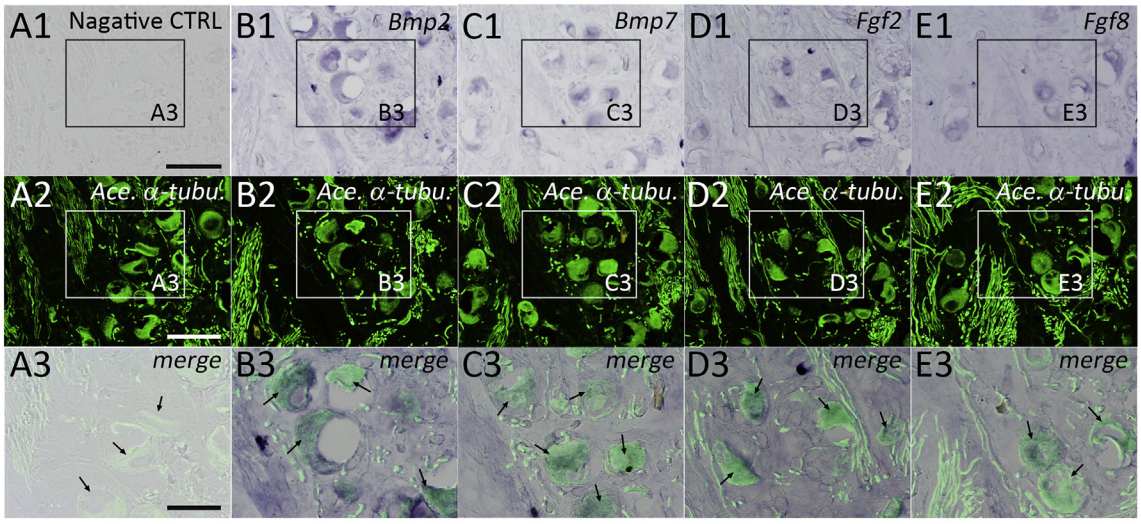

Fig. 5. *Fgf* and *Bmp* gene expression patterns in the trigeminal ganglion. (A1–E1) *In situ* hybridization on the adjacent sections. (A2–E2) Immunofluorescent images. Neural cells were visualized using anti-Acetylated α-tubulin. (A3–E3) Merged images. Arrows indicate the Acetylated α-tubulin cells. (A) Negative control. (B) *Bmp2*. (C) *Bmp7*. (D) *Fgf2*. (E) *Fgf8*. Scale bars in A1, A2, and A3 are 100, 100, and 50 μm, respectively. A1–F1 show the same magnification.

investigated the same gene expression in a denervated gill (Fig. 7G–L). Denervation did not cause any histological damage in the gill (Fig. 7G). Regarding the gene expression pattern, *Bmp2* and *Fgf2* gene expression were much weakened by denervation (Fig. 7I, K). *Bmp7* and *Fgf8* were consistently undetected (Fig. 7J, L). Of note, even *Col1A2* gene expression was weakened by denervation (Fig. 7H), suggesting that broader gene suppression was caused by the denervation in the axolotl gill. These findings imply that *Fgf* and *Bmp* gene expression in nerves and

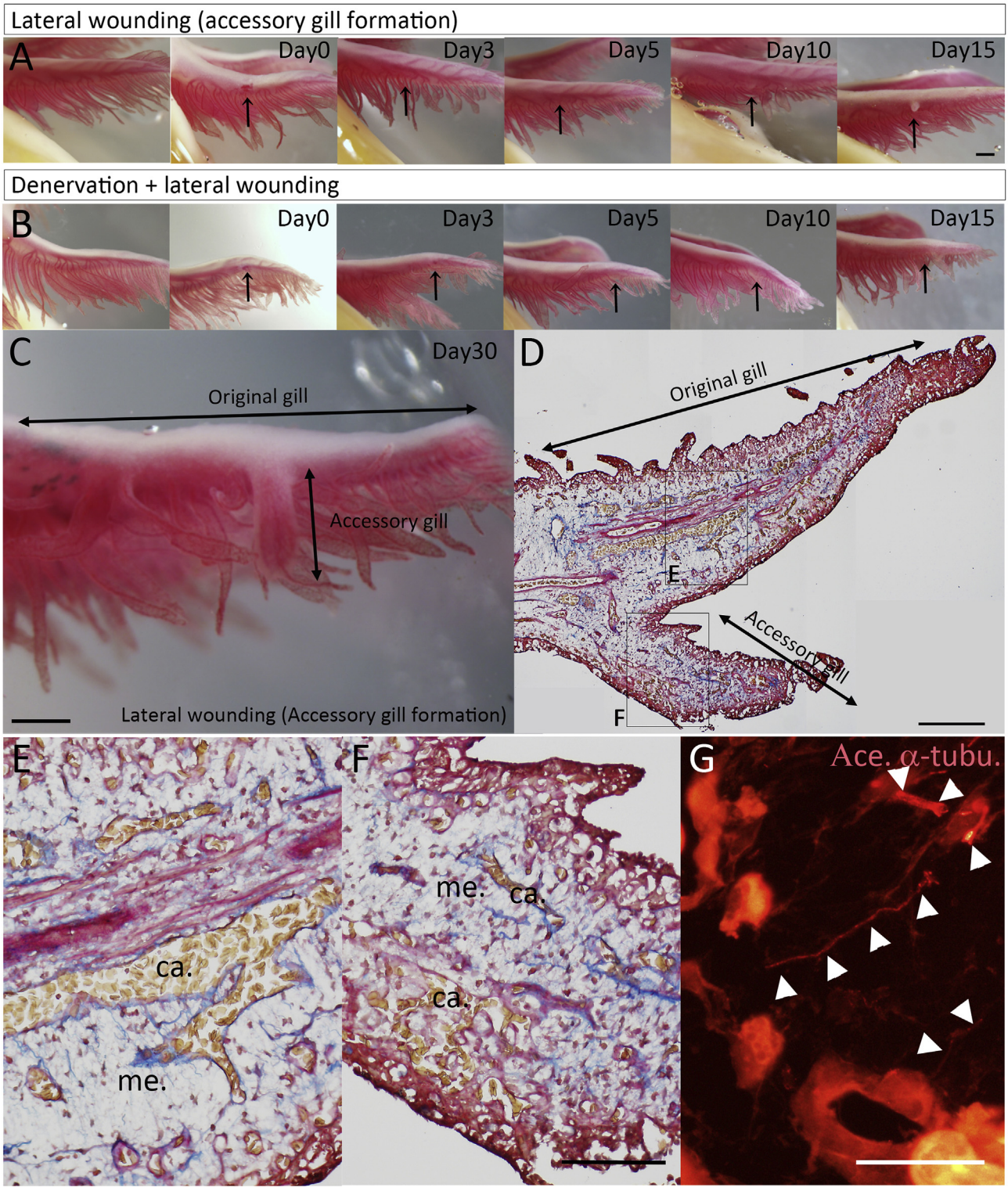

Fig. 6. Accessory gill formation. (A) Accessory gill formation after lateral wounding. A blastema was identifiable on Day 10. Arrows indicate the wounding site. (B) Lateral wounding did not give rise to accessory structures in the denervated gill. Arrows indicate the wounding site. (C) Accessory blastema on Day 30. (D) Histological observation of the induced accessory gill. Trichrome staining was performed. E and F show the higher-magnification views of the boxed regions in D. (G) Axon fibers in the accessory gill. Axon fibers were visualized using immunofluorescence with anti- Acetylated α-tubulin antibody. Arrowheads indicate the axons positive for Acetylated α-tubulin. Scale bars in A, C, D, F, G are 1, 1, 0.5, 0.2, and 0.05 mm, respectively. ca. = capillary. me. = gill mesenchyme.

Table 1
Summary of the surgery on axolotl gills.

	No reaction	Blastema formation	Gill-like formation	total
Lateral wound	1	1	8	10
Lateral wound/Denervation	19	5	0	24
Lateral wound/Denervation/PBS	7	0	1	8
Lateral wound/Denervation/Fgf2+Fgf8+Bmp7	2	7	0	9

mesenchymal tissue plays a role in accessory gill formation coordinately.

Gene expression pattern in a regeneration gill blastema was further investigated (Fig. 8). The blastema was dissected at 10 dpa. In response to the experimental procedures, a large blood blister sometimes formed (Fig. 8A). *Col1A2* was used as a positive control as above (Fig. 8B). *Bmp2* and *Fgf2* expression was similar to that in the intact gill (Fig. 8C, E; Fig. 7C, E). Cells expressing *Bmp7* and *Fgf8* appeared in the mesenchymal region (Fig. 8D, F). We could not determine the cell types of those cells due to a lack of marker genes. The gene expression pattern of a blastema suggests coordinated Fgf and Bmp contribution to gill regeneration from gill mesenchyme and the trigeminal ganglia neurons.

Finally, we investigated whether exogenous Fgf and Bmp can induce a gill blastema (Fig. 9). In this experiment, denervation was performed as shown in Fig. 9A. As expected, denervation prevented the formation of a blastema from a lateral wound in most cases (Fig. 9B, Table 1). The regeneration inducers in limb and tail regeneration have previously been identified (Makanae et al., 2014b, 2016). Bmp2 and Bmp7 showed a

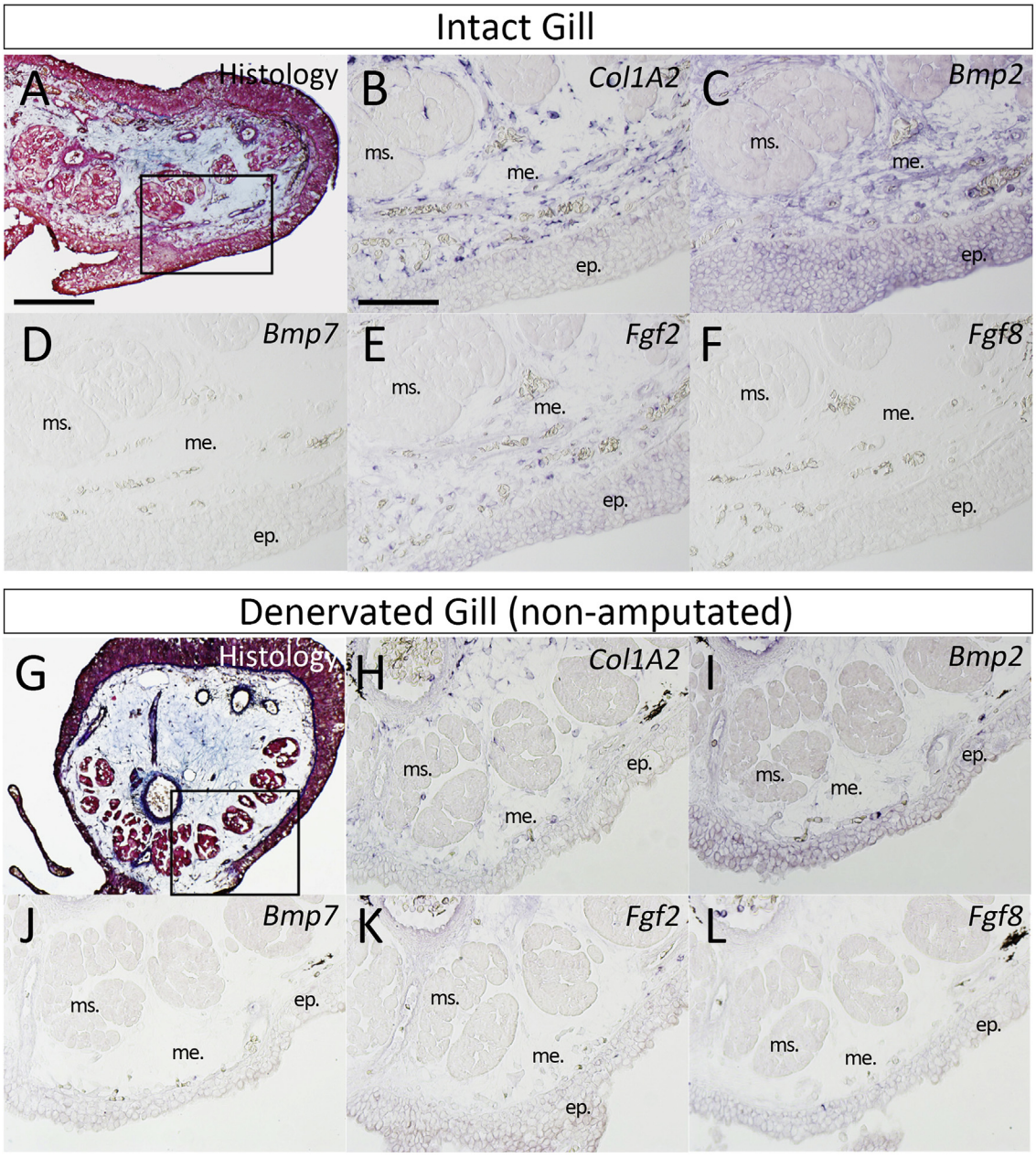

Fig. 7. *Fgf* and *Bmp* gene expression patterns in the axolotl gill. Gene expression patterns were investigated using *in situ* hybridization. (A) Histology of the transverse section of the axolotl gill. (B–F) *In situ* hybridization on the adjacent sections. The approximate region is indicated in A (boxed region). (B) *Type I collagen* (*Col1A2*). (C) *Bmp2*. (D) *Bmp7*. (E) *Fgf2*. (F) *Fgf8*. (G) Histology of the transverse section of the denervated axolotl gill. (H) *Col1A2*. (I) *Bmp2*. (J) *Bmp7*. (K) *Fgf2*. (L) *Fgf8*. Scale bars in A and B are 500 and 200 μm, respectively. A and G a show the same magnification. B–F and G–L show the same magnification. ms. = gill muscle. me. = gill mesenchyme. ep. = gill epithelium.

similar effect on limb and tail blastema induction. For consistency, the same combination of proteins (Bmp7+Fgf2+Fgf8; BFF) was selected in this study. A bead containing BFF was applied to the wound in the denervated gill 3 days after wounding. A blastema was observable in the gills grafted with the BFF bead 5 days after bead grafting (8 days after wounding; Fig. 9C). Histology of the sections revealed mesenchymal cell accumulation over the BFF bead (Fig. 9E, E'). Due to the histological experimental procedures, the induced blastema was shrunk a lot. However, accumulation of the mesenchymal cells over the BFF beads was apparent as compared to gill grafted with the PBS-bead (Fig. 9D and E). Most of the BFF-induced blastemas could not maintain their growth and were resorbed in the end. This might be caused by the limited range of protein diffusion from the grafted bead. These findings suggest that Bmp- and Fgf-signaling are involved in the formation of a gill blastema.

4. Discussion

4.1. Bmp- and Fgf-signaling are involved in gill blastema formation

Bmp2 and *Fgf2* are expressed in the gills of axolotls (Fig. 7) and regulate regeneration responses (Fig. 4). External gills are prone to damage because of their position on the body. Even under laboratory conditions, external gills are easily damaged due to feeding actions by other axolotls. Moreover, the extremal gills are easily affected by water conditions. Due to the high risk of gill loss, axolotls require relatively dynamic homeostasis throughout their life. Thus, cells in a gill would be stochastically in an active state. Mesenchymal expression of *Fgf2* and *Bmp2* may reflect gill condition. Continuous *Bmp2* and *Fgf2* expression may contribute to gill homeostasis in accordance with various water conditions. The importance of Fgf- and Bmp-signaling in gill regeneration is demonstrated in Fig. 4. Chemical inhibition of Fgf- or Bmp-signaling slowed gill regeneration, compared with controls (Fig. 4). However, gill regeneration was not stopped completely by those chemical compounds, suggesting that other signaling mechanisms are involved in gill blastema formation. By contrast, application of Bmp7+Fgf2+Fgf8 into a gill wound was sufficient to induce a blastema in the denervated gills (Fig. 9). This implies that Bmp- and Fgf-signaling are primary regulators for blastema induction.

The source of Bmp and Fgf proteins in amphibian organ regeneration has been explored. In the present study, *Fgf* and *Bmp* gene expression was reported in axolotl gills and gill nerves (Figs. 5 and 7). Therefore, nerves with axons projecting to the gills and the gills themselves may be sources of Fgf and Bmp in gill regeneration. At this time, details on the contribution from those two sources are unknown. However, Fgf and Bmp from nerves may be necessary to overcome the threshold of blastema induction, since denervation inhibited gill regeneration (Fig. 3). In the case of accessory gill formation, simple lateral wounding was sufficient to induce the formation of an accessory gill (Fig. 6A, C). However, denervation prevented such accessory gill formation from lateral wounding (Fig. 6B). This suggests the importance of nerve roles in gill regeneration. Lateral wounding likely damaged some of the axons running from proximal to distal. Some of the damaged axons might be involved in the formation of an accessory gill blastema as shown in Fig. 6G. Existence of axons in a blastema is supported by the presence of axon fibers in the accessory gills. The number of participating axons, however, is likely not high, compared with that in accessory limb formation (Endo et al., 2004; Satoh et al., 2008). Besides, nerves are likely playing a role in maintenance of *Fgf2* and *Bmp2* gene expression in a gill (Fig. 7G–L). Such indirect effects should also be another important factor of nerve regulation in axolotl gill regeneration. Thus, nerves are required to overcome the induction threshold of axolotl gills.

The complexity of the nerve regulation in gill regeneration may be explained by other neural molecules. In limb regeneration, a number of nerve molecules were reported, such as newt anterior gradient (nAG), glial growth factor (GGF), and neuregulin (Pirotte et al., 2016; Satoh et al., 2015). These molecules were reported to play a role in blastema formation in limb regeneration. Given the conserved activity of *Fgf* and *Bmp* genes in limb, tail, and gill regeneration, other nerve molecules likely play a certain role in gill regeneration. Most of the BFF-induced blastemas could not maintain their growth in our experiment (Fig. 9C). The regression of a BFF-induced blastema might be rescued by supplying other nerve molecules. The balance of nerve-supplied molecules may need to be examined in each organ.

4.2. Accessory structures based on regeneration mechanism

Inducing an accessory structure is a major experimental system in urodele amphibians today. The accessory limb model was established

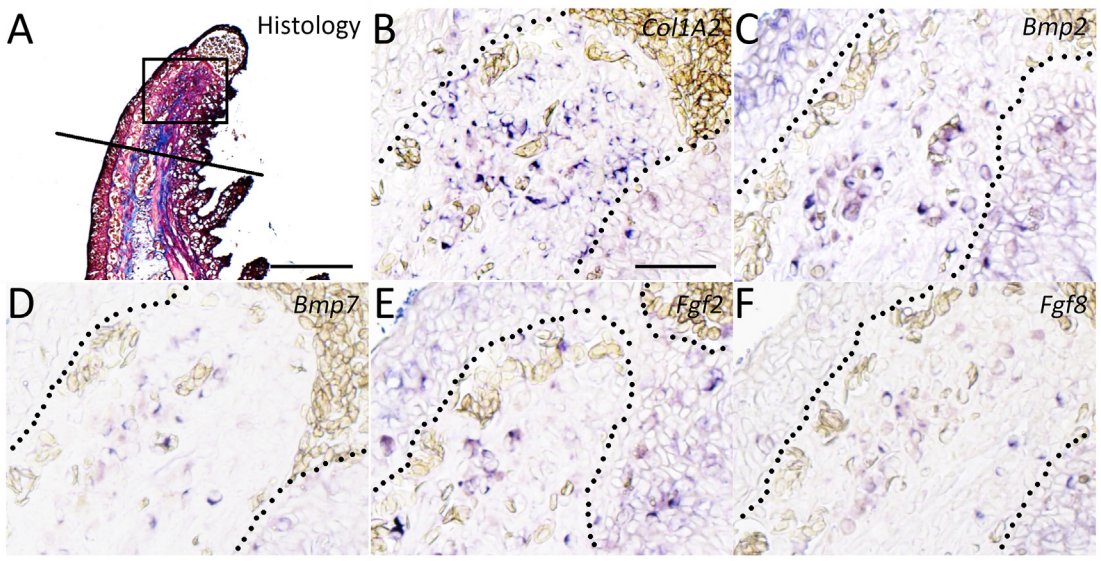

Fig. 8. *Fgf* and *Bmp* gene expression patterns in the regenerating gill of an axolotl. Gene expression patterns were investigated using *in situ* hybridization. (A) Histology of the longitudinal section of the regenerating gill of an axolotl. (B–F) *In situ* hybridization on the adjacent sections. The approximate region is indicated in A (boxed region). (B) *Col1A2*. (C) *Bmp2*. (D) *Bmp7*. (E) *Fgf2*. (F) *Fgf8*. The dotted lines indicate the border of the gill epithelium. Scale bars in A and B are 500 and 100 μm, respectively.

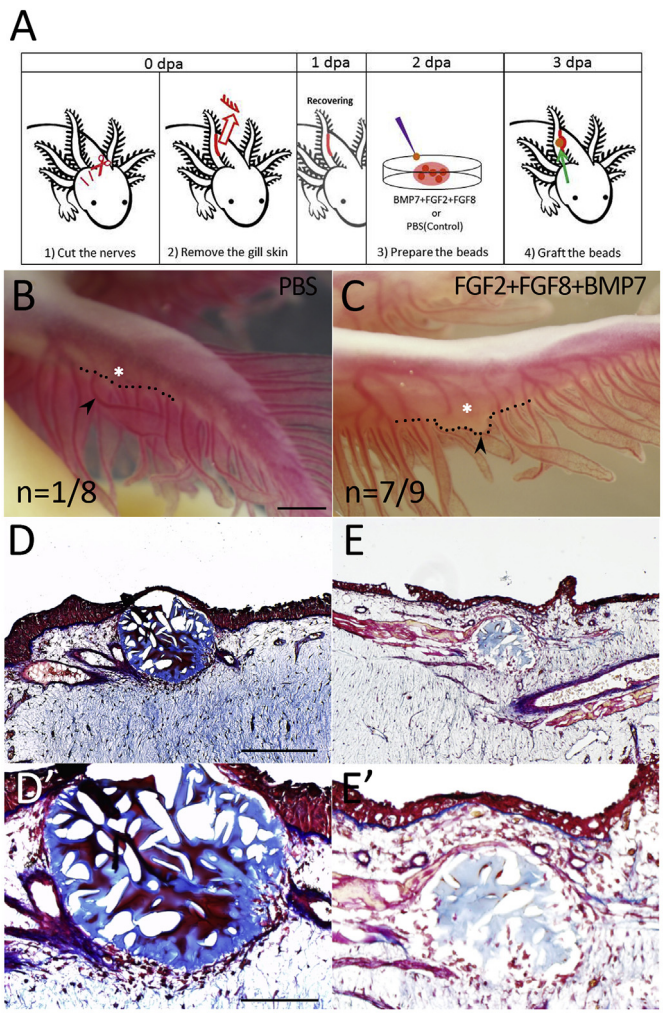

Fig. 9. Accessory blastema formation induced by a cocktail of Bmp and Fgf proteins. (A) Schematic diagram of the surgery. Denervation was performed first. Then, a portion of gill skin was removed. A protein-soaked bead was prepared 1 day before grafting. The bead was placed underneath the newly formed epidermis over the wound. (B) Negative control. A phosphate-buffered saline (PBS)-soaked bead was grafted. No blastema formation was observed. (C) A blastema could be observed after grafting the bead soaked in Bmp7+Fgf2+Fgf8. (D, E) Histological observation. (D) Gill grafted with a PBS-soaked bead. (E) Gill grafted with a Bmp7+Fgf2+Fgf8-soaked bead. Scale bars in B, D, and D′ are 1000, 500, and 200 μm. The asterisks in A and C indicate the location of the grafted bead. The dotted line shows the border of the gill ramus.

first (Endo et al., 2004). Then, an experimental procedure to induce an accessory tail and a gill was established (Makanae et al., 2016, Fig. 6). One of the advantages of the experimental system in which accessory structures are induced is that it allows us to focus on specific tissues in regeneration studies. Organs are always composed of multiple tissue types, and amputation damages all of these tissues, making further analysis difficult. By contrast, the accessory models basically require the creation of a surface wound and the presence of nerves. As shown in Fig. 6A, nerve rerouting was not needed to induce an accessory gill. However, axons penetrate the wound area and likely play a role in blastema induction (Fig. 6A and B). Thus, it appears that fundamental mechanisms are conserved among organs, suggesting that a conserved mechanism regulates the regeneration of appendages in urodele amphibians.

Accessory limb induction requires a skin graft from the contralateral side of a limb (Endo et al., 2015). If the skin wound is created on the anterior side of a limb, the skin graft should be taken from the posterior side. This allows the induction site to have all positional information, as in an amputation plane. An anterior wound can be expected to have dorsal–anterior–ventral positional values, and the skin graft provides the posterior value. Such arrangement of positional values has been considered necessary to advance whole regeneration processes (Makanae et al., 2014b; Nacu et al., 2016). In accessory gill formation, such a skin graft is not needed (Fig. 6A). A gill has positional differences. The dorsal side is covered in smooth skin and the ventral side has gill filaments. There are no apparent differences between the lateral and medial sides. Considering the features of a gill, gill regeneration might require three different values: dorsal, ventral, and anterior or posterior. In limb regeneration, blastema cells were come from within 1 mm from an amputation plane (Currie et al., 2016). Therefore, it is also possible that cells from the contralateral side may be able to migrate to the side of the wound because of the much smaller diameter of a gill, compared with that of a limb. However, it has not been possible to date to investigate such arrangement of positional values no position-specific marker genes have been determined in axolotl gills. It is also worth noting that accessory gill formation was reported using treatment with a chemical component that affects cell positional values (Crawford and Vincenti, 1998). Retinoic acid (RA) is well known as a chemical component that can posteriorize, proximalize, and ventralize limb cells in limb development and regeneration. The effects of RA in gill cells are still unknown. When RA was coinjected with thyroxine (T4) in an animal, metamorphosis was prevented. Accessory gill filaments could be identified among such animals coinjected with RA&T4. The mechanism underlying this phenomenon is still largely unknown. However, the fact that chemical components which can influence positional values in cells can induce accessory gill formation implies that there may be a relationship between the positional values and regeneration. At this moment, the arrangement of positional values in regenerating organs remains an open question. Accessory models may be useful for investigating and understanding positional values.

4.3. *Conserved mechanism of organ-level regeneration*

Axolotl organs are a fascinating target for investigation since they have the ability to regenerate. Much attention has been given to limb regeneration as a representative phenomenon of the massive regeneration ability of axolotls. We have reported that regeneration of a tail and gills is regulated by a mechanism similar to limb regeneration (Fig. 6A, C). Wounding is, no doubt, necessary, and Fgf- and Bmp-signaling are involved in limb, tail, and gill regeneration (Makanae et al., 2014a, 2016, Fig. 5B–E). What does such conservation mean? We speculate that there is a fundamental but total regeneration mechanism at work across a body. Regeneration-competent animals can regenerate some or many of their organs. For example, zebrafish can regenerate the heart, the brain, and fins (Akimenko et al., 1995; Gemberling et al., 2013; Poss et al., 2002). Planarians can regenerate their entire body (Agata et al., 2007). Frogs can start the regeneration process in various organs although the regeneration is usually imperfect (Dent, 1962). By contrast, mammalians basically do not have the ability to regenerate organs even though local or restricted regeneration ability can be found in the liver and distal digits. Thus, the organ-level regeneration ability appears to have disappeared almost at once. The existence of a fundamental regeneration mechanism that controls most organ regenerations could account for the sudden loss of the regeneration ability in various organs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.04.011.

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