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Nerve independent limb induction in axolotls ☆

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ABSTRACT

Urodele amphibians can regenerate their limbs. During limb regeneration, dermal fibroblasts are transformed into undifferentiated cells called blastema cells. These dermis–blastema cells show multipotency. Such so-called endogenous reprogramming of cell differentiation is one of the main targets of amphibian limb regeneration studies. It is well recognized that nerve presence controls the initiation of limb regeneration. Accordingly, nerve factors have been sought in amphibian limb regeneration. To investigate it, a relatively new study system called the accessory limb model (ALM) was developed. Using ALM, two signaling cascades (Fgf and Gdf5 signaling) came under focus. In the present study, Growth and differentiation factor-5 (Gdf5) application to wounded skin initiated limb regeneration responses and resulted in induction of a blastema-like structure in the absence of a nerve. However, the Gdf5-induced structure showed defects as a regeneration blastema, such as absence of detectable *Prrx*1 expression by *in situ* hybridization. The defects could be remedied by additional Fibroblasts growth factor (Fgf) inputs. These two inputs (Gdf5 and Fgfs) were sufficient to substitute for the nerve functions in the induction of limb regeneration. Indeed, Fgf2, Fgf8, and Gdf5 applications with the contralateral skin graft resulted in limb formation without nerve supply. Furthermore, acquisition of cartilage differentiation potential of dermal fibroblasts was tested in an *in vivo* and *in vitro* combination assay. Dermal fibroblasts cultured with Gdf5 were difficult to participate in cartilage formation when the cultured cells were grafted into cartilage forming region. In contrast, dermal fibroblasts cultured with Fgf2 and Fgf8 became easier to participate into cartilage formation in the same procedure. These results contribute to our understanding of molecular mechanisms of the early phase of amphibian limb regeneration.

Introduction

Urodele amphibians have remarkable regeneration capacity compared with higher vertebrates. They can regenerate missing body parts, such as limbs. Recent studies have revealed that organ/tissue regeneration is observable in mice, but regeneration capability in urodele amphibians is incomparably higher (Han et al., 2005; Peyton et al., 2012; Porrello et al., 2011). Several studies have been conducted to gain knowledge from urodele amphibians for application to organ damage in higher vertebrates. Despite this effort, the mystery of the regeneration ability of urodele amphibians remains unsolved.

In axolotl limb regeneration, undifferentiated cells, called blastema cells, are formed after limb amputation. Induction of the blastema cells is the major mystery in the study of amphibian limb regeneration. The blastema, an aggregation of undifferentiated cells, is formed on the amputation plane. Although the definition of blastema cells is still vague, the words "blastema cells" are generally used to describe cells present in a blastema, except for axons and hematocytes. Recent studies have shown that a blastema is a heterogenetic cell population (Kragl et al., 2009). Muscle-derived blastema cells are actually myogenic and do not show multipotency, whereas dermis-derived blastema cells are multipotent (Gardiner et al., 1986; Hirata et al., 2010; Kragl et al., 2009; Maden and Wallace, 1976; Muneoka et al., 1986) and can participate in forming dermis, cartilage, and other connective tissues. Dedifferentiation of differentiated dermal fibroblasts takes place at the very beginning of limb regeneration. Such dedifferentiation can be considered an endogenous reprogramming of cell differentiation. The dedifferentiation mechanism that generates undifferentiated blastema cells remains an unsolved issue.

A new experimental system, called the accessory limb model (ALM), was recently established (Endo et al., 2004; Satoh et al., 2007). ALM is now an alternative study system in amphibian limb regeneration (Makanae and Satoh, 2012). ALM studies elegantly show that rerouting nerves and skin wounding are sufficient for

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induction of limb regeneration, meaning that a blastema can be induced by nerves and a skin wound. After skin wounding, migrating epidermis, called wound epidermis/epithelium (WE), begins to cover the wound immediately after surgery (Carlson et al., 1998; Satoh et al., 2008b). In the absence of nerves, interaction between WE and underlying mesenchymal cells leads to regular wound healing, resulting in scarless healing (Levesque et al., 2010). In the process, migration of fibroblasts toward the wound site can be observed; this is similar to that observed in other higher vertebrates. Such migrating fibroblasts can be regarded as activated fibroblasts because they are migrating and dividing to reconstitute a dermis. Thus, dermal collagen deposition and reorganization follow immediately after fibroblast migration (Satoh et al., 2008a, 2012). In contrast, the deviation of a nerve to the skin wound results in blastema induction (Endo et al., 2004). WE becomes thickened and does not develop its basement membrane, preserving WE–blastema mesenchyme interaction (Bryant et al., 1971; Neufeld et al., 1996). Such regeneration-specific WE is called the apical epithelium/epithelial cap (AEC) and is considered a structure functionally similar to the apical ectodermal ridge (AER) in limb development of higher vertebrates (Christensen and Tassava, 2000; Nye et al., 2003). AEC are considered essential structures for limb regeneration (Thornton, 1957). AEC and nerves are believed to create a regenerative environment for dermal fibroblasts. Several molecules expressed in AEC and nerves have been identified (Endo et al., 2000; Nye et al., 2003; Satoh et al., 2008b, 2011; Yokoyama et al., 2000). Fgfs are commonly expressed in both, indicating that Fgfs secreted from both contribute to the regenerative environment. After the AEC–nerve interaction, dermal fibroblasts migrate to the wound site and receive regeneration-specific inputs from the surrounding environment. Dermal fibroblasts then undergo dedifferentiation to an undifferentiated state, called blastema cells. Blastema cells accumulate and divide *in situ*, resulting in a dome-shaped ALM blastema (Endo et al., 2004; Makanae and Satoh, 2012). For the ALM blastema to continue growing and patterning, all positional values of a limb must be determined prior to blastema formation (Makanae and Satoh, 2012). Surgically, this determination is achieved by grafting a piece of skin from the contralateral side from the wounded region to supply the missing positional value (s). When a skin wound is created in the anterior side of a limb, the anterior wound can be expected to have anterior, dorsal, and ventral positional values, and missing posterior positional values. If the skin graft from the contralateral side (posterior) of the limb is supplied to the anterior wound, the wound supplied with the posterior skin graft can be expected to have all anteroposterior and dorsoventral values. Although such arrangement of the positional values seems essential for limb pattering, it is still possible to induce an ALM blastema without providing the missing positional value(s). In that case, the ALM blastema, called a "bump," is shrunken but still shows the same features as a regular blastema (Endo et al., 2004; Satoh et al., 2007). The ALM blastema expresses some blastemal marker genes, shows cartilage differentiation ability, and can participate in regular limb regeneration (Satoh et al., 2007). Thus, an ALM blastema without a skin graft can still be considered equivalent to a regular blastema. Accordingly, for the study of blastema induction, an ALM blastema with or without a skin graft can be used instead of a regular blastema, meaning that just two of many tissue types are sufficient for the study of early regulation of limb regeneration. Furthermore, ALM allows focusing on only these two types of tissues when induction of limb regeneration is investigated (Satoh et al., 2010b). Thus, ALM can be considered a much simpler system for the limb regeneration study than an amputated limb.

The main objective of an amphibian limb regeneration study is to reveal the molecular regulation of induction of a regeneration blastema. Fgf signaling has been investigated for this purpose. Fgfs are secreted from nerves, AEC, and blastema cells as mentioned above (Han et al., 2001; Mullen et al., 1996; Poulin et al., 1993; Satoh et al., 2011; Yokoyama et al., 2001, 2000; Zenjari et al., 1996). Activation of Fgf signaling can be expected to begin from a very early stage of limb regeneration. Recently, it was demonstrated that application of Fgf2 and Fgf8 to wounded skin could trigger blastema formation in ALM (Satoh et al., 2011). This result implies that Fgf signaling regulates cellular dedifferentiation of limb fibroblasts. Fgf signaling may play an important role in the regulation of blastema induction. However, it may not be the sole factor. Some candidates were already reported. For example, inhibition of Tgf-β signaling resulted in a failure of induction of a regeneration blastema (Levesque et al., 2007). And Anterior gradient (AG) protein can substitute for nerves and controls blastema induction (Kumar et al., 2007). Thus, early regulation in blastema induction has begun to be revealed.

Table 1
Limb induction in aneurogenic condition.

Experiment	Total injuries	AL formation*	Rate of AL induction (%)
Fgf2+Fgf8+Skin graft	8	0	0
Gdf5+Skin graft	8	0	0
Fgf2+Fgf8+Gdf5+Skin graft	12	5	41.7

* AL=Accessory limb.

We were performing comprehensive and comparative analyses using ALM and wounded skin to find additional inducers with Fgf2 and Fgf8. This was because Fgf2 and Fgf8 were not sufficient to induce a limb even though it was sufficient to induce a blastema (Satoh et al., 2011; Table 1). Candidate molecules were selected from the analysis. Among them, we focused on Gdf5 signaling. Gdf5, also called *Bmp*14 and *Cdmp*1, is a protein encoded by BMP family genes. We found that Gdf5 application to wounded skin resulted in induction of a blastema-like "bump." However, the bump structure was not identical to that of a regeneration ALM blastema, given that the bump cells did not show the cartilage differentiation ability when grafted into a cartilage-differentiating area. Moreover, the Gdf5-induced bump showed a different gene expression profile than a regular regeneration ALM blastema. Additional Fgf signaling inputs induce blastema formation from the Gdf5-induced bump formation. Such a Fgf- and Gdf5-induced blastema showed features similar to those of a regular blastema, including cartilage differentiation capability. These results indicate that Gdf5 signaling attracts fibroblasts to a wound site and that Fgf signaling induces regenerative responses in these cells.

Materials and methods

Animals and surgery

Animals of nose-to-tail length 8–12 cm were obtained from private breeders and housed in aerated water at 22 °C. Their limbs, which had never previously been subjected to surgery, were used for ALM surgery. Surgical procedures were performed as described previously (Endo et al., 2004; Makanae and Satoh, 2012).

Beads grafting

Gelatin beads were made following the previously described way (Satoh et al., 2011). These beads can be used as protein sustained-released beads. Air-dried beads were allowed to swell in the solutions. Stock solution (1 μg/μl) was prepared following the