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Reactivation of larval keratin gene (*krt62.L*) in blastema epithelium during *Xenopus* froglet limb regeneration

Akira Satoh[a,*], Kazumasa Mitogawa[a], Nanami Saito[a], Miyuki Suzuki[b], Ken-ichi T. Suzuki[b], Haruki Ochi[c], Aki Makanae[a]

[a] *Okayama University, Research Core for Interdisciplinary Sciences (RCIS), 3-1-1, Tushimanaka, Kita-ku, Okayama 700-6230, Japan*
[b] *Hiroshima University, Department of Mathematical and Life Sciences, Graduate School of Science, 1-3-1, Kagamiyama, Higashihiroshima, Hiroshima 739-8526, Japan*
[c] *Institute for Promotion of Medical Science Research, Yamagata University, Faculty of Medicine, 2-2-2 Iida-Nishi, Yamagata-shi, Yamagata 990-9585, Japan*

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ABSTRACT

Limb regeneration is considered a form of limb redevelopment because of the molecular and morphological similarities. Forming a regeneration blastema is, in essence, creating a developing limb bud in an adult body. This reactivation of a developmental process in a mature body is worth studying. *Xenopus laevis* has a biphasic life cycle that involves distinct larval and adult stages. These distinct developmental stages are useful for investigating the reactivation of developmental processes in post-metamorphic frogs (froglets). In this study, we focused on the re-expression of a larval gene (*krt62.L*) during *Xenopus* froglet limb regeneration. Recently renamed *krt62.L*, this gene was known as the *larval keratin* (*xlk*) gene, which is specific to larval-tadpole stages. During limb regeneration in a froglet, *krt62.L* was re-expressed in a basal layer of blastema epithelium, where adult-specific keratin (Krt12.6.S) expression was also observable. Nerves produce important regulatory factors for amphibian limb regeneration, and also play a role in blastema formation and maintenance. The effect of nerve function on *krt62.L* expression could be seen in the maintenance of *krt62.L* expression, but not in its induction. When an epidermis-stripped limb bud was grafted in a froglet blastema, the grafted limb bud could reach the digit-forming stage. This suggests that *krt62.L*-positive froglet blastema epithelium is able to support the limb development process. These findings imply that the developmental process is locally reactivated in an postmetamorphic body during limb regeneration.

1. Introduction

Most amniotes do not possess the ability to regenerate organs. Organ regeneration has, however, been observed in some amphibians, and this remarkable ability has fascinated scientists for a long time. To understand this phenomenon and to pioneer novel medical treatments, the superior organ regeneration ability observed in these amphibians has been investigated.

A regeneration blastema is a key structure for successful limb regeneration. After limb amputation, the epidermis around the amputation plane can migrate to cover the amputation surface (Ferris et al., 2010; Satoh et al., 2008), and this process is thought to contribute to preventing infection. The covering epithelium is called wound epithelium/epidermis (WE). WE can interact with underlying tissues and cells (Makanae and Satoh, 2012; Nye et al., 2003). In the absence of nerves, dermal collagens are deposited under WE, leading to a wound healing process (Satoh et al., 2012). In the presence of nerves, collagen deposition is suppressed and an accumulation of cells takes place instead (Satoh et al., 2012). These accumulating cells are called blastema cells, and the dome-like structure is called a regeneration blastema (Goss, 1969). Regeneration-incompetent animals cannot form a blastema after limb amputation. Therefore, the blastema induction mechanism is key for successful limb regeneration. Blastema cells are undifferentiated cells, some of which are multipotent (Hirata et al., 2010; Kragl et al., 2009; Muneoka et al., 1986). The origin of blastema cells is still controversial. It is apparent, however, that mature full-thickness skin gives rise to multipotent blastema cells (Hirata et al., 2010; Kragl et al., 2009; Muneoka et al., 1986). It has been thought that differentiated dermal cells dedifferentiate through an interaction between the nerves and the blastema epithelium (Makanae and Satoh, 2012; Nye et al., 2003). Thus, the blastema induction process involves the induction of multipotent cells in an adult body.

* Corresponding author.
E-mail address: satoha@cc.okayama-u.ac.jp (A. Satoh).

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Recent studies have unveiled the molecular mechanisms of blastema induction in amphibians (Makanae et al., 2013, 2014; Satoh et al., 2011). The accessory limb model was developed as an alternative experimental model to study amphibian limb regeneration (Endo et al., 2004; Mitogawa et al., 2014). In the accessory limb model, a small square shaped wound is created by removing full thickness skin on one side (*e.g.* the anterior side). Then, a piece of skin graft is prepared from the contralateral side of a limb (*e.g.* the posterior side) and grafted into the skin wound. Such a skin wound results in simple skin wound healing without scarring. When nerves are rerouted to the skin wound, a blastema is induced. The induced blastema has full competency and can grow a limb, resulting in an additional limb called an accessory limb. This model had been used to identify nerve molecules that can induce a blastema in amphibians. The grafting of Fgf2- and Fgf8-soaked beads into wounded skin resulted in blastema formation without the presence of a nerve (Satoh et al., 2011). An Fgf-induced blastema has ordinary blastema features. An Fgf-induced blastema cannot, however, complete the entire limb regeneration process and eventually regresses (Satoh et al., 2011). Cooperative Bmp7 (or Bmp2) application with Fgf2 and Fgf8 resulted in induction of the entire limb regeneration process (Makanae et al., 2014, 2016). The induced blastema can complete the limb regeneration process from wounded skin. Moreover, it has already been demonstrated that Bmp7+Fgf2+Fgf8 application can induce limb and tail regeneration in multiple organisms (Makanae et al., 2016). After determining the nerve factors responsible for blastema induction, the detailed mechanism of blastema induction is now being investigated at the molecular level.

A regeneration blastema has been considered to be akin to a developing limb bud. In fact, a similar gene expression pattern can be observed in a developing limb bud and a regenerating blastema. For instance, *Lmx1b* is a dorsally expressed gene in a developing limb bud (Cygan et al., 1997). *Lmx1b* is expressed in limb bud mesenchyme from an early limb developmental stage. In *Lmx1b* mutant mice, all tissues of the limb are affected, resulting in a biventral limb pattern; this is particularly apparent in the distal-most portion of the limb, the autopod (Chen et al., 1998). This dorsal-restricted *Lmx1b* expression pattern is re-created in a regenerating limb blastema (Makanae and Satoh, 2012; Matsuda et al., 2001; Satoh and Makanae, 2014). *Shh* is expressed in the posterior side of a developing limb bud (Echelard et al., 1993; Tickle and Towers, 2017; Torok et al., 1999) and a regenerating limb blastema (Imokawa and Yoshizato, 1997; Torok et al., 1999), regulating anteroposterior limb patterns. Furthermore, when a developing limb bud was transplanted into a regenerating blastema, the grafted cells could participate in the host regeneration (Muneoka and Bryant, 1982, 1984). Hence, creating a regeneration blastema is akin to reactivating a limb developmental process locally within an adult body.

Xenopus can provide a reasonable experimental environment to assess whether the reactivation of a developmental process takes place in an adult body. This is due to their unique lifespan, which is divided by metamorphosis. Premetamorphic *Xenopus* tadpoles develop limb buds, and limb development is completed during the tadpole stages. More importantly, the expression of some *keratin* genes is drastically turned on/off before and after metamorphosis (Suzuki et al., 2009). *Larval-specific keratin* (*krt62.L*; synonym: *xlk*, *krt5.5*) is only expressed in the basal layer of the epidermis during the larval and tadpole stages, and its expression is shut down in an adult body (Watanabe et al., 2001). Conversely, *adult-specific keratin* (*krt12.6.S*; synonym: *xak-c*) expression is limited to adult epidermis (Watanabe et al., 2002). Therefore, the *keratin* gene expression pattern is an ideal indicator to assess the issue of reactivation of a developmental process in an adult body during limb regeneration.

The present study focused on *krt62.L* expression in *Xenopus* froglet blastemas. *Xenopus* froglet, a young and small frog, can grow a blastema after limb amputation. However, the blastema cannot form a well-patterned limb (Dent, 1962). Instead, a cone-shaped structure, called a spike, is formed. Although an induced blastema is unable to form a well-patterned limb, the blastema induction process is nerve-dependent, and Fgf- and Bmp-signaling can substitute for the nerve role, which is similar to the process seen in “regenerative” urodele amphibians (Satoh et al., 2015). Therefore, it is reasonable to investigate blastema induction processes in *Xenopus* froglets. Some *keratin* genes are commonly used as epidermal marker genes. Recently, *Xenopus keratin* genes were re-arranged and re-named (Suzuki et al., 2016). *Xlk* is now referred to as *krt62.L*, and *xak-c* as *krt12.6.S*. In the present study, we found that *krt62.L* became detectable in a regenerating *Xenopus* blastema but not in intact skin. *Fgf8*, which is another limb-bud–specific marker gene, was also detected in the same expression domain as *krt62.L*. Krt12.6.S expression remained limited to blastema epithelium. In addition, *krt62.L* reactivation was not dependent on nerve presence, although its maintenance was dependent on nerve presence. Consistent with the reactivation of larval genes in a regenerating blastema, a froglet blastema could support limb bud development when a developing limb bud was grafted into a regenerating blastema. Our findings may help to understand the reactivation of the developmental process in the adult body during organ regeneration and in dedifferentiation during limb regeneration.

2. Materials and methods

2.1. Animals and surgical procedures

Froglets (*Xenopus laevis*; 2–3 cm from nose to cloaca) were obtained from a breeder (*Xenopus* Fukuoka, Fukuoka, Japan) and housed in aerated water at 22 °C. A transgenic *X. laevis*, *Tg(eef1a1.L:H2B-GFP)*, was produced using the following process. The H2B-GFP (gift from Geoff Wahl, Addgene plasmid # 11680) was cloned into the downstream of the e*ukaryotic translation elongation factor 1 alpha 1* (*eef1a1.L*) promoter (Johnson and Krieg, 1994; Kanda et al., 1998). A DNA fragment of *eef1a1.L:H2B-GFP* was integrated into the genome using the sperm nuclear transplantation method with oocyte extracts (Kroll and Amaya, 1996).

The animals were anesthetized prior to all surgical and fixation procedures using 0.05% MS222 (Sigma, St. Louis, MO, USA). For limb bud mesenchyme grafting, tadpoles were selected based on the developmental stage (Nieuwkoop and Faber, 1994). Limb buds were collected from st. 52 tadpoles. The distal half of the collected limb bud was used. The epidermis was removed from the collected distal limb buds. The epidermis was easily removed from the limb mesenchyme using treatment with 0.05% EDTA/ phosphate-buffered saline (PBS) at room temperature for 5 min. The isolated limb bud mesenchyme was placed into a middle bud stage blastema using forceps and needles (Fig. 6A). The middle bud stage blastemas could usually be obtained 10–14 days after limb amputation. The skin around the border of the amputation plane was cut and a tunnel was created up to the distal tip using very fine forceps. The graft, an epidermis-removed st. 52 limb bud, was placed in the distal region through the tunnel. It was important to put the graft just underneath the blastema epithelium.

2.2. FGF inhibitor treatment

SU5402 (Calbiochem, San Diego, CA, USA) was dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) to prepare a 10 mM stock solution. For the inhibitor treatment experiment, we kept the animals, whose limbs had been amputated 10 days before, in the presence of SU5402 (10 μM) in water. The water was refreshed every day. After 4 days of treatment, the animals were harvested.