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A diffusible signal derived from hematopoietic cells supports the survival and proliferation of regenerative cells during zebrafish fin fold regeneration

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ABSTRACT

Multicellular organisms maintain body integrity by constantly regenerating tissues throughout their lives; however, the overall mechanism for regulating regeneration remains an open question. Studies of limb and fin regeneration in teleost fish and urodeles have shown the involvement of a number of locally activated signals at the wounded site during regeneration. Here, we demonstrate that a diffusible signal from a distance also play an essential role for regeneration. Among a number of zebrafish mutants, we found that the zebrafish *cloche* (*clo*) and *tal*1 mutants, which lack most hematopoietic tissues, displayed a unique regeneration defect accompanying apoptosis in primed regenerative tissue. Our analyses of the mutants showed that the cells in the primed regenerative tissue are susceptible to apoptosis, but their survival is normally supported by the presence of hematopoietic tissues, mainly the myeloid cells. We further showed that a diffusible factor in the wild-type body fluid mediates this signal. Thus, our study revealed a novel mechanism that the hematopoietic tissues regulate tissue regeneration through a diffusible signal.

Introduction

When tissues are injured, animals regenerate the lost or wounded parts to maintain the tissue integrity by the mechanism of tissue homeostasis, which is fundamental to multicellular organisms, but still poorly understood. In particular, many teleost fish and urodeles can regenerate limbs, tail, and fins through a process known as the epimorphic regeneration, whereas mammals are unable to regenerate lost appendages such as limbs. Unraveling the mechanism underlying the complete tissue regeneration process will help us understand the principles of tissue homeostasis and contribute to regenerative medicine.

Classical studies have shown that epimorphic regeneration involves specialized cell types, wound epidermis and the blastema. The blastema, which is induced in the underlying mesenchyme of the injured site, is a mass of proliferating cells that contribute to the principal parts of reconstituted tissues. The wound epidermis, a thick epidermis that covers the regenerating tissue, has also been thought to play an important role for regeneration (Campbell and Crews, 2008). During the past decade, studies in adult zebrafish fin regeneration have started to reveal the molecular basis of regeneration. They revealed a number of regeneration-response genes (Padhi et al., 2004; Schebesta et al., 2006) and necessary signals such as Fgf (Poss et al., 2000), Hedgehog (Quint et al., 2002), Bmp (Smith et al., 2006), Wnt (Stoick-Cooper et al., 2007), Activin (Jaźwińska et al., 2007), Igf (Chablais and Jazwinska, 2010), retinoic acid (Blum and Begemann, 2012), Notch (Grotek et al., 2013; Munch et al., 2013), and reactive oxygen species (Gauron et al., 2013).

In addition to the conventional regeneration model using adult zebrafish fin, the regeneration model using larval fin fold has been developed (Kawakami et al., 2004) and enabled us to reveal additional and complementary molecular basis (Mathew et al., 2007; Yoshinari et al., 2009; Ishida et al., 2010). Moreover, the larval fin fold model has an advantage in that genetic mutants can be tested for their regeneration phenotypes, because many zebrafish lethal mutants can survive for a relatively long period (Yoshinari et al., 2009).

Significantly, the molecules and signals so far studied in adult and larval regeneration models were the locally expressing ones that are activated in response to tissue traumas. On the other hand, it has not been known whether or not tissue regeneration could be influenced by factor(s) that are provided by other tissues away from the injured site.

In the present study, we found that the zebrafish mutant, *cloche*, which has defects in most hematopoietic and endothelial

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cells (Stainier et al., 1995), displayed a unique regeneration defect that apoptosis occurs in the regenerative tissue. We show that the process of regeneration is initiated in the mutant; however, the primed regenerative cells do not proliferate, but undergo apoptosis. We further showed that the hematopoietic tissues, in particular the myeloid lineage cells, are essential for the survival of regenerative cells. We provided evidence that the survival of regenerative cells is normally supported by a diffusible factor that exists in the body fluid of wild-type larvae, but not that of mutant.

Materials and methods

Fish husbandry and fin amputation

Zebrafish (*Danio rerio*) were maintained in accordance with the Animal Research Guidelines at Tokyo Institute of Technology. Zebrafish were maintained in a re-circulating system with a 14 h (hrs)/day and 10 h/night cycle at 28.5 °C. Fin fold amputation was performed as previously described (Kawakami et al., 2004). For wound healing assay, the larval fin fold was punctured with a fine glass capillary (tip diameter, 20 μm). After amputation, larvae were incubated in egg water (0.06% artificial marine salt, 0.0002% methylene blue) and subjected to the respective analyses. The zebrafish strains used in this study are wild-type strain, TL, and mutant strains, *tal1*^{t21384}, *tp53*^{zdf1}, *vlad tepes*^{vltm651}, *clo*^{m39}, and *clo*^{la1164}.

Fish genotyping

Genotype of *tal1* mutant was determined according to the previously described protocol (Bussmann et al., 2007). Genotyping of *tp53*^{zdf1} was performed according to the protocol supplied by the Zebrafish International Resource Center (ZIRC) using primers (TPA03: 5'-ACATGAAAT TGCCAGAGTATGTGTC-3'; TPA04: 5'-TCGGATAGCC-TAGTGCGAGC-3'). The PCR products were digested with MboII and analyzed the restriction length polymorphism. Genotype of *cl*^{m39} was determined by the PCR using the linked markers, *z*1496 and/or *z*8617.

Whole-mount in situ hybridization (ISH) analysis

Whole-mount ISH analysis was performed according to the protocol by Thisse and Thisse (2008). After detection of ISH signal, the samples were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for color preservation, equilibrated with 80% glycerol, and mounted on slide glasses for inspection and taking photographs. The ISH probes used in this study were previously described (Yoshinari et al., 2009)

Whole-mount immunohistochemistry

Zebrafish larvae were fixed with 4% PFA in PBS at 4 °C for overnight, washed three times with PBS supplemented with 0.1% Triton X-100 (PBTx), dehydrated with methanol, and stored at −30 °C. Samples were rehydrated with PBTx and blocked with the blocking buffer (5% serum and 0.2% bovine serum albumin in PBTx) for 2 h at room temperature (RT). Subsequently, the samples were reacted with anti-DsRed antibody (1:1000 dilution; Clontech) at 4 °C for overnight. Following extensive washing with PBTx (10 min × 6 times) at RT, the samples were incubated with Alexa555-conjugated secondary antibodies at 4 °C for overnight. After washing with PBTx, the tail regions were isolated and mounted in 80% glycerol containing 2.5% 1,4-diazabicyclo [2,2,2] octan (Nacalai Tesque) as an anti-fading reagent. Fluorescent pictures were acquired by a confocal microscopy.

Chemical inhibitors

The DNA polymerase inhibitor, Aphidicolin (Wako), was used at 100 μM. The VEGF receptor inhibitor, PTK787 (vatalanib; JS Research Chemical Trading; Chan et al., 2002), was used at 5 μM. The ROS inhibitors, VAS2870 (Sigma; Niethammer et al., 2009) and apocynin (Merck; Love et al., 2013), were used at 5 and 100 μM, respectively. The JNK inhibitor, SP600125 (Tocris; Ishida et al., 2010), was used at 10 μM. The inhibitors were dissolved in dimethylsulfoxide as stock solutions (at least 1000 × concentrations of respective working concentrations) and stored at −30 °C. Inhibitors were diluted with egg water before use. PTK787 was applied at 10 h post fertilization. Other inhibitors were applied to zebrafish larvae at least 1 h before fin fold amputation.

Cell proliferation analysis

Proliferating cells were labeled with 5-Bromo-2-deoxyuridine (BrdU; 5 mM). The larvae were fixed with 4% PFA for 2 h at RT, dehydrated with methanol and stored at −30 °C. Immunochemical detection was performed as described previously (Yoshinari et al., 2009). The BrdU-labeled cells were quantified from the acquired confocal images.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Larvae were fixed with 4% PFA at 4 °C for overnight, dehydrated with methanol, and stored at −30 °C. Detection was performed using an *in situ* apoptosis detection kit (Roche). Briefly, the samples were rehydrated with PBTx, treated with 10 μg/ml Proteinase K in PBTx for 3 min at RT, washed with PBTx, and refixed with 4% PFA in PBS for 20 min. The samples were further incubated in a freshly prepared 0.1% sodium citrate, 0.1% Triton X-100 on ice for 15 min, washed with PBTx, and reacted with the TUNEL reaction mixture at 37 °C for 1 h. The reaction was terminated by washing with PBTx. The samples were mounted under 80% glycerol and observed under a fluorescence microscope.

Sudan Black B staining for neutrophils

Larvae were fixed with 4% formaldehyde in PBS for 1 h at RT, rinsed with PBS, and incubated in 0.03% Sudan Black B (Sigma). After extensive washing with 70% ethanol and rehydration with PBS+0.1% Tween 20, samples were mounted with 80% glycerol.

Injection experiments

Injections were performed according to a standard procedure. Fertilized zebrafish eggs were dechorinated with 2% pronase (Roche) and used for microinjection during the 1- to 4-cell stages. Antisense morpholino oligonucleotides (MO) used in this study are:

tnnt2a-MO (5′-CATGTTTGCTCTGATCTGACACGCA-3′);
*pu.*1-MO (5′-GATATACTGATACTCCATTGGTGGT-3′);
*tal*1-MO (5′-AATGCTCTTACCATCGTTGATTTCA-3′);
standard control (std) MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′).

For mRNA injection, the mRNA was synthesized *in vitro* from the linearized plasmid, pCS2-*EGFP-bcl2* (Langenau et al., 2005), using the mMessage Machine kit (Ambion), and injected at 1–4 cell stages (0.5 nl of 0.5 μg/μl solution) into fertilized eggs obtained by incrosses of *clo* heterozygous fish.

Cell transplantation

Wild-type or *clo* heterozygous fish carrying the transgene *Olactb:* loxP-DsRed-loxP-EGFP (Yoshinari et al., 2012) was mated

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