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Effects of light illumination and the expression of *wee1* on tissue regeneration in adult zebrafish

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ABSTRACT

The zebrafish exhibits an enhanced capability of regenerating most of its adult tissues. In this study, we examine the roles of light illumination and functional expression of mitosis-specific gene *wee1* on adult zebrafish caudal fin regeneration after amputation. During the first 3 days post-amputation (dpa), the caudal fin regenerate rapidly in the day but slowly at night when the fish are kept in a normal light–dark cycle (LD) condition. However, this day–night rhythm of fin regeneration is not seen when the fish are kept in constant dark (DD), constant light (LL), or in fish in which the circadian rhythms are disrupted by random light (RL) exposures. The rate of fin growth reaches the peak levels at 2.5 dpa in LD, but is delayed when the fish are kept in DD, LL or RL conditions. In zebrafish in which the expression of *wee1* is blocked by morpholinos, regeneration of the caudal fin is affected. Interestingly, the expression of *wee1* also displays robust circadian rhythms. Together, the data suggests that fin regeneration in zebrafish is regulated by both environmental cues and functional gene expressions. Alterations in lighting conditions or inhibition of *wee1* expression result in decreases in fin regeneration after injury.

1. Introduction

Previous studies on tissue repair or regeneration engaged a variety of animal models, including rat, bird, frog and fish [1–7]. Among them, the zebrafish (*Danio rerio*) is a particularly suitable model for studying tissue regeneration in adult animals. The zebrafish exhibits an enhanced capability of regenerating most of its adult tissues, including retina, spinal cord, kidney, heart, liver and fin [8–24]. In zebrafish, fin tissues rapidly regenerate after injury. Initiation of fin regeneration (epidermal cells migration, accumulation, mesenchymal cells reorganization and proliferation) may occur within a few hours after injury [25–29]. By three weeks post amputation, fin tissues are fully regenerated. Regenerated fins appear nearly identical to their original length, shape, pattern and color, which is likely due to genetic controls or cell lineage determinations [30–33].

In a recent study, Shao et al. [34] reported that zebrafish have unlimited potential in fin regeneration, i.e., the fish are capable of regenerating injured fins after repeated surgeries and in different ages. During the first 7 dpa, the rate of fin regeneration and the expression of fin growth marker genes (such as *msxb*, *fgf201* and *bmp2b*) are similar in animals that receive single or multiple fin amputations (up to 10 repeated surgeries at 48-h intervals) and in animals that are between 4 and 28 months old. Fin regeneration may be influenced by different factors, e.g., colony densities, temperatures and pH values. In zebrafish, for example, fin regeneration is faster in 33 °C than in 28 °C [25]. Fin regeneration is also affected by exposures to chemical or radioactive compounds, for example, ionizing radiation induces rapid fin growth [13], whereas tetrachlorodibenzo-*p*-dioxin decreases fin regeneration [35].

In this research, we examined caudal fin regeneration in adult zebrafish under different lighting conditions, e.g., in fish that were kept in normal light–dark cycles (LD), constant light (LL), constant dark (DD), or after random light exposures (RL). We also measured fin growth on different days post fin amputation in zebrafish in which the expressions of early circadian genes (*clock* and *per1*) were interrupted and the expression of cell-mitosis regulatory gene *wee1* was inhibited by morpholinos injections. Our results suggest that fin regeneration in zebrafish is regulated by both environmental factors and functional gene expressions.

2. Materials and methods

2.1. Fish care and fin amputation

AB zebrafish (*Danio rerio*; between 5 and 8 months old) were used in this study. The fish were kept in circulating system water (distilled water with Instant Ocean salt) and were fed twice a day with freshly hatched brine shrimp. Normally, the fish were

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kept in the cyclic light–dark conditions (room fluorescent light, from 7 am to 9 pm). The fish were anesthetized with 0.1% tricaine prior to the surgery. Approximately 50% of the caudal fin was amputated using a razor blade. The fish were allowed to recover in tanks set at 33 °C [25]. For the DD experiments, the fish were kept in the dark starting at 9 pm in the previous night, and maintained in the dark thereafter. For the LL experiments, the fish were placed in the light at 7 am in the previous day, and maintained in the light conditions thereafter. For the RL experiments, the fish were kept in DD for 7 days but randomly exposed to light (room light, 15–20 min per exposure).

2.2. Morpholino injections

Morpholinos (MOs) were designed according to the 5′ sequence of the UTR of *wee1* gene [36,37]. MO sequences were 5′-CGTGTCTCCCAGCCCCGAAACTCAT-3′. MOs (conjugated with GFP) were diluted in Daniean buffer at the final concentration of 0.1 mM. Immediately after fin amputation, MOs were injected (0.5–1.0 μl) to the dorsal side of the caudal fin followed by electroporations [19]. Control fish received sham-injections with either Daniean buffers or mis-matched MOs (substitution of 5 nucleotides in *wee1* MOs) to the dorsal side of the caudal fin. At 3 dpa, we measured the length of the regenerating caudal fin (the third bony ray from dorsal side and third bony ray from ventral side, respectively). The effects of MO injections on fin growth were determined by comparing the length of third bony ray of dorsal caudal fin (where MOs were injected) and the length of third bony ray of ventral caudal fin (no injections, which served as internal controls). We divided the length of third bony ray of dorsal caudal fin over the length of third bony ray of ventral caudal fin. If the D/V ratio (length of dorsal caudal fin/length of ventral dorsal fin) was smaller than 0.9, it indicated that regeneration of dorsal caudal fin was decreased (i.e., inhibition of *wee1* expression).

2.3. Quantitative RT-PCR

Total RNA was extracted from caudal fins (for each PCR, 4 fish were used) using Trizol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) and was reverse-transcribed by M-MLV reverse transcriptase (Promega, Madison, WI) using the oligo (dT) primers. Primer sequences included: *clock*, Forward 5′-ACCCTC ACCCAGACTACCTAA-3′, Reverse 5′-ACCGTCGCTATGAAACACACTC-3′; *per1*, Forward 5′-AGAGGGAGAACGATGGACG-3′, Reverse 5′-CT GGTTGGTCACTGTTGGAT-3′; *wee1*, Forward 5′-CATCGCCACGG AAAGTC-3′; Reverse 5′-TGGGGGGTATCAAAAAGAC-3′; β-actin, Forward 5′-TTCACCACCACAGCCGAAAGA-3′, Reverse 5′-TACCGCAAG ATTCCATACCCA-3′. RT-PCR conditions included a denaturing step at 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s for real time plate read, and a final extension at 72 °C for 5 min. β-Actin was used for data normalization.

3. Results

3.1. Caudal fin regeneration in different lighting conditions

We examined caudal fin regeneration after amputation in zebrafish that were kept in different lighting conditions (normal light–dark cycle, LD; constant dark, DD; constant light, LL; random light exposures, RL). In all the conditions examined, during the first few days post amputation (dpa), the rate of fin growth rapidly increased. In fish that were kept in the LD conditions, the rate of fin growth reached the peak level at 2.5 dpa, whereas in fish that were kept in other conditions (DD, LL or RL), the rate of fin growth reached the peak levels until 4 dpa. In all conditions examined, fin growth maintained at the peak level for 1–2 days, and then gradually decreased (Fig. 1A–D). In zebrafish that were kept in LD, during the first 3 dpa the caudal fin regenerated rapidly in the day but slowly at night (Fig. 1A). No obvious day–night rhythms in fin growth were detected in zebrafish that were kept in DD, LL or RL conditions (Fig. 1B–D).

We measured the length of the regenerating caudal fin on different days post amputation. In LD, the caudal fin regenerated approximately 0.5 ± 0.1 mm by 3 dpa. It grew to 1.3 ± 0.2 mm by 7 dpa, which is approximately 70% of its original length measured before amputation. The caudal fin was fully regenerated by 18–20 dpa (Fig. 2). In zebrafish that were kept in DD, the rate of regeneration was decreased in the first few days after surgery in comparison to the rate of fin regeneration in LD. At 3 dpa, in DD conditions the caudal fin regenerated approximately 0.25 ± 0.2 mm. In LL, by contrast, steady increases in fin growth were observed in the first few days after amputation. By 3 dpa, the caudal fin regenerated nearly 0.6 ± 0.1 mm in LL. By 7 dpa, regenerating caudal fin was 0.9 ± 0.2 and 1.4 ± 0.2 mm, respectively, in fish that were kept in DD or LL. In both conditions, the caudal fin was fully regenerated by 18–20 dpa, which is similar to fin regeneration observed in zebrafish that were kept in LD (Fig. 2).

3.2. Caudal fin regeneration was slightly decreased when the circadian rhythms were

In regenerating caudal fin, the expression of early circadian genes (such as *clock* and *per1*) fluctuates between the day and night. In DD, for example, the expression of *clock* mRNA was low in the day but high in the evening. Between 10 am and 10 pm, the expression of *clock* increased 12-fold (Fig. 3A). The expression patterns of *per1* were opposite to the expression of *clock*. In DD, high expression of *per1* was seen in the early morning, but the expression gradually decreased during the day and in the evening. From 10 am to 4 am, *per1* mRNA expression decreased 16-fold (Fig. 3B).

We examined fin regeneration in zebrafish in which the circadian rhythms were interrupted light random light exposures (RL, 15–20 min per exposure, room fluorescent light applied during the day and night while the fish were otherwise kept in DD). After 7 days random light exposures, the circadian rhythms of *clock* and *per1* expressions were completely interrupted, i.e., the expression of *clock* was similar between the day and night, and no differences in *per1* expression were detected between early and mid-mornings. Under these conditions, fin growth was slightly decreased (Fig. 1D). At 3 and 7 dpa, for example, the regenerating caudal fin grew to 0.4 ± 0.1 and 1.1 ± 0.2 mm, respectively. In RL, the injured caudal fin was fully regenerated between 18 and 20 dpa (Fig. 2).

3.3. Functional expression of wee1 is required for proper fin regeneration

The expression of *wee1* has been shown to play important roles in cell mitosis and proliferation [38–40]. In regenerating caudal fin, the expression of *wee1* mRNA rhythmically fluctuated during a 24-h period. In LD, the expression was low in the early morning, increased steadily during the day and peaked in the late afternoon. In the evening, the expression of *wee1* mRNA began to decrease. The expression of *wee1* mRNA reached the lowest level in the early morning hours (Fig. 3C). This patterns of *wee1* expression were seen in zebrafish kept in DD (Fig. 3D), suggesting the involvement of endogenous circadian clocks in the regulation of *wee1* mRNA expression.

To evaluate the roles of functional expression of *wee1* in caudal fin regeneration, we examined caudal fin growth in zebrafish in which the expression of *wee1* was inhibited by injection of *wee1*-specific morpholinos (MOs) (Fig. 4). We injected GFP-tagged *wee1* MOs to the dorsal side of the caudal fin immediately after

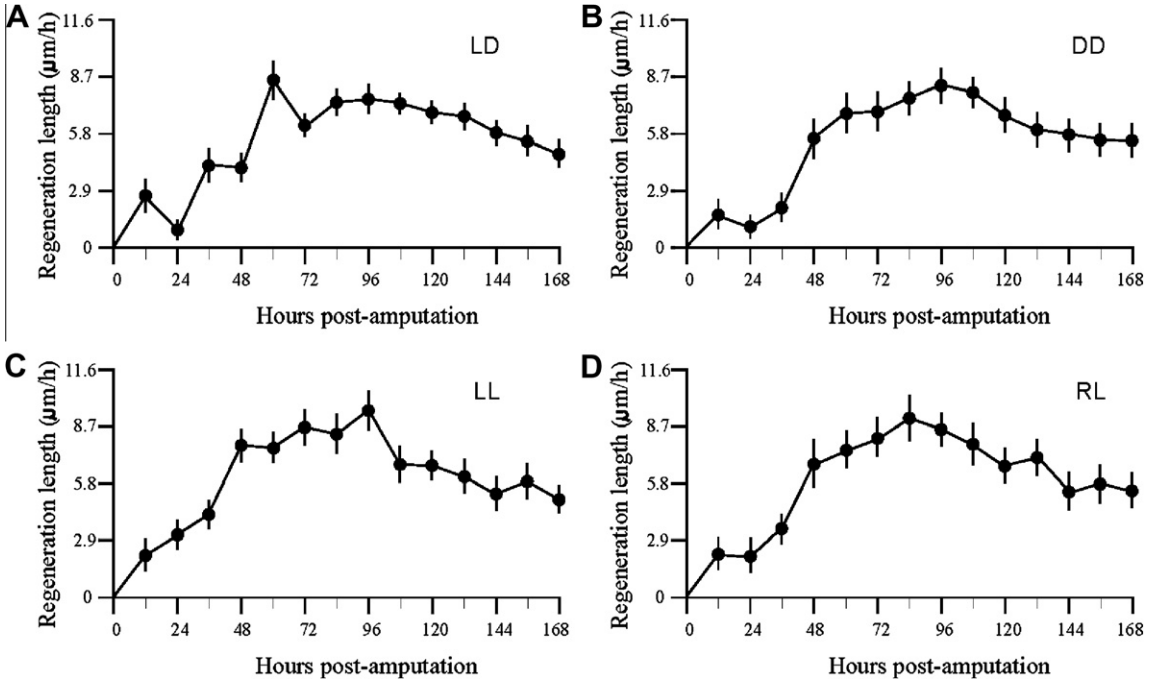

Fig. 1. Caudal fin regeneration (fin growth per hour) in the first 7 dpa in zebrafish that were kept in different lighting conditions ($n = 12$ in each condition). In all the conditions examined, the rate of fin growth increased in the first 3 dpa, and then gradually decreased. In LD, fin growth was rapid in the day (with light illumination) but was slow at night (without light illumination). Data represent the means ± SE. LD, normal light–dark cycle; DD, constant dark; LL, constant light; RL; random light exposures.

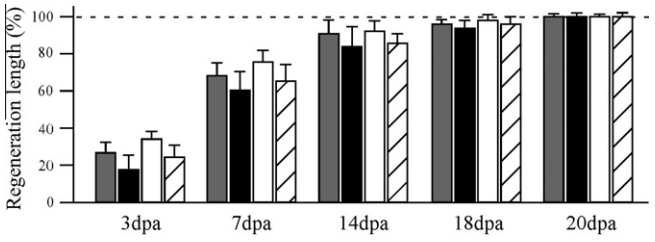

Fig. 2. Percentage of regenerating caudal fin to its original length on different days after amputation in zebrafish that were kept in different lighting conditions ($n = 12$ in each condition). The caudal fin fully regenerated by 18–20 dpa in all the conditions examined. Although no statistic differences ($p > 0.05$ in all cases) were detected in fin growth under different conditions, there were tendency that the caudal fin grew rapidly in fish that received light illumination (e.g., in LL, LD, RL) but slowly in fish that were kept in the dark (DD). Data represent the means ± SE. Gray bars, fish in LD; black bars, DD; white bars, LL; hatched bars, RL.

fin amputation. At 3 dpa, we compared the length of regenerating dorsal caudal fin and the length of regenerating ventral caudal fin. In control fish (injection of mis-matched MOs or Daniean buffer to dorsal caudal fin), we observed a small number of fish (9 out of 41 examined) that showed slight decreases in regeneration of dorsal caudal fin in comparison to regeneration of ventral caudal fin, i.e., the D/V ratio (the length of dorsal caudal fin divided by the length of ventral caudal fin) was smaller than 0.9. This was probably due to mechanic damages to fin tissues by the injection procedures. In zebrafish that received *wee1* MO injections, a large number of fish (11 out of 24 fish examined) showed decreased dorsal caudal fin regeneration in comparison the ventral caudal fin regeneration. This suggests that functional expression of *wee1* is required for proper fin regeneration.

4. Discussion

In this study, we examined the effects of lighting illumination and the expression of cell mitosis-specific gene *wee1* on caudal fin regeneration in adult zebrafish. We found slight decreases in fin growth during early stages of regeneration (i.e., the first 3 dpa) in fish that were kept DD in comparison to fin growth in animals kept in LD (Fig. 1). This suggests that proper light illumination may play a role in fin regeneration. The underlying mechanism of light on fin regeneration remains to be studied, but it may relate to functional expression of *wee1*, which is shown to be regulated by a circadian mechanism and light illumination. In the mid-morning (LD or DD), the expression of *wee1* mRNA steadily increased (Fig. 3C, D). In LD, between 7 am (normal light-on time) and 10 am, the expression of *wee1* increased by nearly 100%. In DD, however, between 7 am and 10 am, the expression of *wee1* increased less than 50%. This suggests that in addition to the intrinsic circadian control, light illumination promotes *wee1* expression. Because *wee1* plays important roles in cell mitosis and proliferation (38–40), it is conceivable that light illumination promotes tissue regeneration by activating *wee1*-mediated signaling pathways. Noted also is that in LD, during the first 3 dpa, fin regeneration occurred rapidly in the day and slowly at night. In LL, by contrast, steady increases in fin growth were seen during the day and night. Together, the data suggests that light illumination promotes tissue regeneration.

We demonstrated that whether or not the zebrafish maintains its biological rhythms may not be critical on caudal fin regeneration. In LD or DD, the expression of early circadian gene (i.e., *clock* and *per1*) in regenerating caudal fin displayed robust day–night rhythms. Random light exposures (in fish that were kept in DD) effectively interrupted the circadian rhythms of *clock* and *per1* mRNA expressions, but the rate of fin generation in RL only slightly decreased in comparison to the rate of fin generation in control animals kept LD. Because the expression of *wee1* is also influenced by the circadian clocks, we suspect that the decreases in fin growth after random light exposures may be due to the interruption of functional expression of the *wee1* gene. When the expression of *wee1* was inhibited (by anti-*wee1* MOs), decreases in fin regeneration were observed. We were unable to evaluate the effects of *wee1* inhibition on the entire process of fin regeneration (i.e., up to

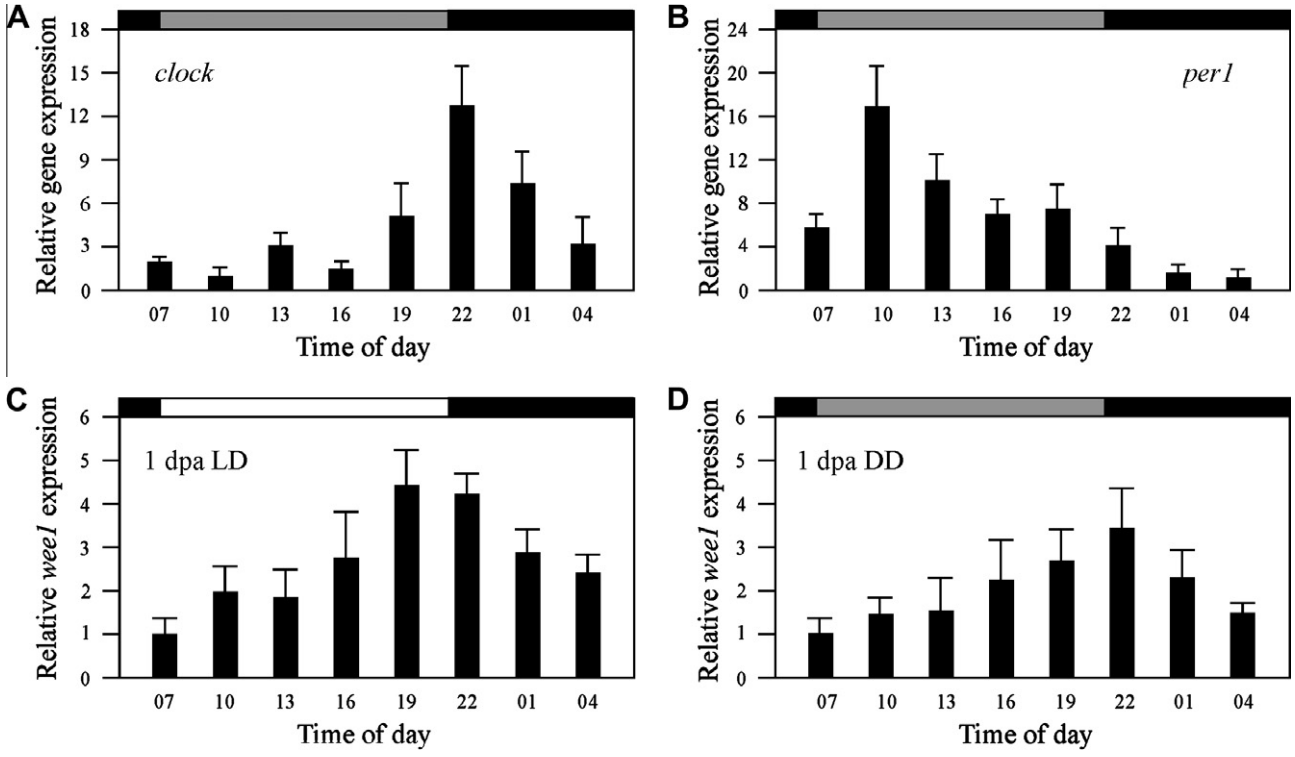

Fig. 3. Expression of *clock*, *per1* and *wee1* mRNA in regenerating caudal fin isolated from fish kept in DD or LD. (A,B): Relative *clock* and *per1* mRNA expression in the day and night while the fish were kept in DD. The lowest expression of *clock* (at 10 am) and *per1* (at 4 am) was normalized to 1, respectively. (C,D): Expression of *wee1* mRNA in LD or DD conditions in the first day after amputation. Under both lighting conditions, the lowest level of expression at 7 am was normalized to 1. The horizontal bars represent the lighting conditions: black bars, night without light; gray bars, subjective day without light; white bar, day time with light. Data represent the means ± SE ($n = 8$). $p < 0.05$.

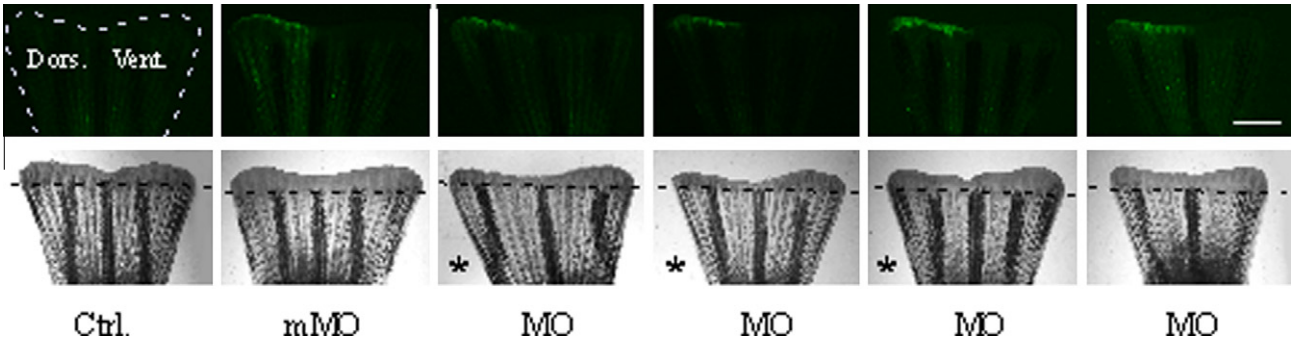

Fig. 4. Fluorescent- and bright-light images of regenerating caudal fin at 3 dpa after injection of *wee1* MOs, mis-matched MOs or Daniean buffer (to the dorsal side of caudal fin). The experiments were conducted in the normal LD conditions. Horizontal dashed lines indicate the amputation sites. Asterisks indicate the fish that showed decreased regeneration of dorsal caudal fin in comparison to regeneration of ventral caudal fin. Ctrl., control fish that received Daniean buffer injections; mMO, mis-matched MO injections; MO, *wee1* morpholino injections. Dors., dorsal; Vent., ventral. Scale bar, 1 mm.

20 dpa), because the effects of MO on gene translation persist only approximately 5 days [19]. Repeated MO treatments did not seem feasible, because the injection and electroporation procedures may cause damages to fin tissues.

In all the conditions examined, the caudal fin fully regenerated (to its original length) after 18–20 dpa. Zebrafish that were kept in LL seemed to fully regenerate the caudal fin most rapidly (Fig. 2). At 18 dpa, for example, 8 out of 12 fish examined in the LL condition showed full regeneration of the caudal fin, whereas only 3 out of 12 fish showed full regeneration when they were kept in DD. Together, our data suggests that environmental cues (i.e., light illumination) may influence fin growth particularly during early stages of regeneration, whereas intrinsic mechanisms, i.e., genetic control of cell proliferation and differentiation, play critical roles in the regulation of fin growth after injury.

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