Pattern regulation in the stripe of zebrafish suggests an underlying dynamic and autonomous mechanism

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The mechanism by which animal markings are formed is an intriguing problem that has remained unsolved for a long time. One of the most important questions is whether the positional information for the pattern formation is derived from a covert prepattern or an autonomous mechanism. In this study, using the zebrafish as the model system, we attempted to answer this classic question. We ablated the pigment cells in limited areas of zebrafish skin by using laser irradiation, and we observed the regeneration of the pigmentation pattern. Depending on the area ablated, different patterns regenerated in a specific time course. The regenerated patterns and the transition of the stripes during the regeneration process suggest that pattern formation is independent of the prepattern; furthermore, pattern formation occurs by an autonomous mechanism that satisfies the condition of ''local self-enhancement and long-range inhibition.'' Because the zebrafish is the only striped animal for which detailed molecular genetic studies have been conducted, our finding will facilitate the identification of the molecular and cellular mechanisms that underlie skin pattern formation.

local self-enhancement and long-range inhibition | pigment patterns | reaction–diffusion mechanism

Animals exhibit a wide spectrum of pigment patterns. Although it has been attracting considerable interest for long time (1, 2), the mechanism underlying the emergence of these patterns remains largely unknown.

In his pioneering paper in 1952 (3), Alan Turing showed that various spatial patterns arise in a system in which two substances react and diffuse at different rates. This system is now commonly termed as the reaction–diffusion (RD)[¶] system, and it has become the standard for theoretically studying of biological pattern formation (4, 5). To form the stationary patterns, the system needs to satisfy a necessary condition, "local self-enhancement and long-range inhibition" (6–9). Recent molecular genetic experiments corroborated the existence of such condition during the events in biological pattern formation (9–11).

Using computer simulations, theoretical studies have shown that a variety of characteristic animal skin patterns can be reproduced by RD mechanism (12–15). Nevertheless, the similarity of the patterns made by the computer simulation is not enough to prove that the RD mechanism underlies the pigment pattern (16, 17). Therefore, it is desired to identify the molecular network that controls the pigment pattern formation.

Recent studies have focused on the stripe pattern of zebrafish as the model system to investigate the mechanism of animal pigmentation (18–20). Many genes related to the pigmentation of zebrafish have been identified (21–27), and investigations on the functions of these genes are underway (28–33). However, a strong theoretical premise is necessary to develop a complex molecular network that can explain the pattern formation. If it is shown that the RD mechanism underlies the stripes of zebrafish, the mechanism can be used as the working hypothesis to investigate the network.

A characteristic property of the stationary patterns generated by RD mechanism is the ability to self-regulate the pattern and their robustness against perturbations (34). For instance, in numerical simulations, if enlargement of the field is introduced by increasing the number of cells in which the reaction occurs, the stripes or spots do not enlarge accordingly; in fact, the intrinsic size is retained by the splitting or insertion of new stripes or spots (1). Such dynamic rearrangement was really observed during growth in the skin of the growing marine angelfish *Pomacanthus imperator* (35). This observation strongly suggested that the stripe patterning of this angelfish is based on an autonomous mechanism such as an RD system. Unfortunately, in the case of the zebrafish, the stripes do not show such dynamic rearrangements during the course of the growth of the fish. New stripes formed during body growth are simply added to the outermost stripe (19, 36). However, if the RD mechanism also controls the pigment pattern of zebrafish, it should be possible to induce some dynamic rearrangement by introducing an artificial perturbation.

By using a pulse laser system with an attached microscope, we were able to ablate two types of pigment cells, namely, black melanophores and yellow xanthophores, without causing any serious damage to the other cells in and around the target region. As suggested by molecular genetic studies (32, 37–40), these pigment cells play a major role in pigment pattern formation, and if so, the loss of these pigmented cells in a defined region would disturb the stationary "pattern."

Based on the above assumption, we first ablated all of the pigment cells in a wide region of the body trunk to erase the entire "pattern," and then observed the regeneration process to test whether the zebrafish stripes are independent of any prepattern. Subsequently, to observe the dynamic nature of the pattern, we observed the transition of the stripes in the area adjacent to the ablated region. Finally, we demonstrated that the time course and final regenerated patterns correspond precisely to those expected if the underlying mechanism is assumed to be RD.

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Abbreviation: RD, reaction–diffusion.

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¶Although we used the regular term ''RD mechanism'' in this article, it should be noted that the ability to generate patterns is restricted to a specific class of reactions; for example, those in which self-enhancement is coupled with a long-ranged antagonistic effect (6).

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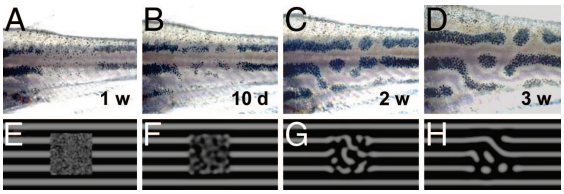

Fig. 1. Regeneration process of the stripes of both zebrafish and computer simulation. (*A–D*) Regeneration of stripes without an inherent prepattern in zebrafish. Images were recorded after 1 week (*A*), 10 days (*B*), 2 weeks (*C*), and 3 weeks (*D*) after ablation of both melanophores and xanthophores in the wide region above the anal fin base. At the beginning of the regeneration, melanophores and xanthophores were randomly distributed in the ablated area (*A*). Then, each type of pigment cell became to segregate their distribution (*B* and *C*), and the stripe pattern without original anterior–posterior direction was regenerated (*D*). Note that this regeneration was achieved by autonomous migration of pigment cells. (*E–H*) Time-lapse captured images of the regeneration process outputted by computer simulation. We used the Gierer–Meinhardt model (6, 8), which is one of the activator–inhibitor type of RD models, for the simulation. Equations and the details for simulation are available in *Materials and Methods* (*Computer Simulations*). The white indicates an area where a concentration of the assumptive activator (*u* in the equations) is the highest, whereas the black shows the highest concentration area of assumptive inhibitor (*v* in the equations). Parameters were selected to facilitate the stripe formation. The parameter *c*, which causes saturation of self-enhancement of the assumptive activator in the equation, is crucial to stripe formation (8). As the initial condition for pattern regeneration, we set a horizontal stripe pattern in the 256 × 128 field. For the ablation, the central region was replaced by a random pattern. Captures were produced after 500 (*E*), 1,000 (*F*), 3,000 (*G*), and 10,000 (*H*) iterations, respectively.

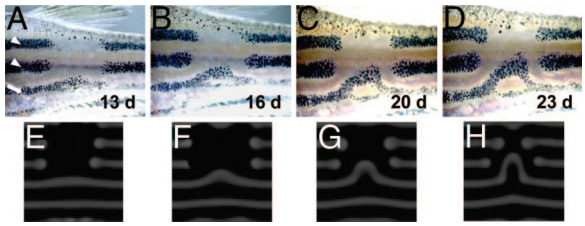

Fig. 2. Rearrangement process of the ventralmost stripe of both zebrafish and computer simulation. (*A–D*) Pattern rearrangement induced by partial ablation of the stripes in zebrafish. Images were captured at 13 (*A*), 16 (*B*), 20 (*C*), and 23 (*D*) days after ablation of the melanophores in the dorsalmost and the central black stripes (arrowheads). Although new melanophores frequently appeared in the ablated area during the first week of experimentation, these were ablated around 24-h intervals. Subsequently, the development of new melanophores within this region became less frequent. As a result, this region was filled with only xanthophores, and the remaining ventralmost stripes (arrow) began to curve and move upward into the area (*A–D*). (See also SI Movie 1.) (*E–H*) Computer simulation of the stripe rearrangement. Used equations are identical with that used in Fig. 1. Horizontal stripes were set as the initial pattern in the 80 × 80 field. The white indicates an area where a concentration of the assumptive activator (*u* in the equations) is the highest, whereas the black shows the highest concentration area of assumptive inhibitor (*v* in the equations). Subsequently, with the ''paint tool,'' which was used to erase the contents of the target region, we manually erased a part of the stripes to form a large region where the activator is absent. Simulation captures were produced after 1,000 (*E*), 2,000 (*F*), 3,000 (*G*), and 4,000 (*H*) iterations, respectively [see the details of the simulation in *Materials and Methods* (*Computer Simulation*)].

Results

Experiment 1: Regeneration of Stripes Without an Inherent Prepattern. We used young 20-day-old fish for the ablation experiment. At this age, the primary formation of the pigment stripes is complete. By using laser irradiation, we ablated all of the melanophores and xanthophores on the left side of the body trunk above the anal fin base; subsequently, we observed their regeneration process. Approximately 24 h after laser ablation, all of the pigment cells in the region were visibly dead, and the pigment debris was released into the hypodermis (data not shown). Within 3 days, the debris disappeared, and the ablated region became pigment-free. Approximately 1 week after the ablation, several melanophores and xanthophores appeared in a random manner throughout the ablated area (Fig. 1*A*). Approximately 10 days after the ablation, the regenerated pigment cells began to segregate (Fig. 1*B*). Subsequently, regions in which either melanophores or xanthophores were predominant emerged. Gradually, the delineation between the different cellular regions became more prominent (Fig. 1 *B* and *C*), and a clear striped pattern was regenerated ≈3 weeks after ablation (Fig. 1*D*). The width of the resultant stripes was similar to the original one. However, the stripes displayed a variable orientation (Fig. 1*D*). This regenerated stripe pattern was relatively stable regardless of its orientation and permanently maintained.

To simulate the regeneration of the stripe pattern, we used the Gierer–Meinhardt model of RD equations (6, 8), which is one of the activator–inhibitor type of the RD model (details are available in *Materials and Methods*). The sequence of pattern regeneration that was predicted by using the simulation (Fig. 1 *E–H*) was similar to the regeneration pattern observed in live zebrafish (Fig. 1 *A–D*) both in its process and resultant pattern. In both the live zebrafish and simulation, the regenerated stripes retained the original spacing (Fig. 1 *D* and *H*). However, in contrast to the standard anterior–posterior direction of the unaffected stripes, the orientation of the regenerated stripes was more or less arbitrary (Fig. 1 *D* and *H*).

Experiment 2: Pattern Rearrangement Induced by Partial Ablation of the Stripes. Another specific property of the stationary pattern formed by the RD mechanism is the dynamic regulation of the pattern (34). Therefore, if the original stripe width was artificially changed, the adjacent stripes are expected to orient themselves to recover the original width. To test this hypothesis, we selectively ablated the melanophores in the dorsalmost and the central black stripes (arrowheads in Fig. 2*A*). During the first week of experimentation, new melanophores frequently appeared in the ablated region; these were ablated around 24-h intervals. Because of the continual elimination of melanophores, the xanthophores gradually developed and filled the region; furthermore, the development of new melanophores within this region became less frequent. Eventually, a large area was filled with only xanthophores (Fig. 2*A*).

At the beginning of the experiments, the ventralmost black stripe (arrow in Fig. 2*A*) comprised a narrow strip of melanophores that thickened during the first week [supporting information (SI) Movie 1]. This early states were identical to that observed in the control fish. However, during the second week, the remaining ventralmost stripes began to curve and move upward toward the dorsal region (Fig. 2 *A* and *B*). This moving stripe formed a bell-shaped pattern that filled the vacant space (Fig. 2 *C* and *D*). The shape and width of stripe remained permanently (SI Movie 1). This experiment was performed on three individuals. In the other two cases, the ventralmost stripe moved dorsally and connected with the anterior terminal of the caudal central stripe, and the width of the stripe remained the same (SI Movies 2 and 3). In all cases, the rearranged patterns were permanent and quite stable.

The equations and parameters used for the simulation in this experiment were identical to those used in the first simulation (details are available in *Materials and Methods*). Horizontal stripes were set as the initial pattern and erased the contents of the target region. The results observed for the simulation (Fig. 2 *E–H*) were quite similar to those observed in the real experiment (Fig. 2 *A–D*).

Discussion

Stripes are one of the common patterns displayed by many animals (41). The zebrafish pattern is especially interesting because complete and detailed molecular genetic data regarding their patterns are expected to be available in the near future (18, 42–45). In contrast to the stripes on the tropical marine-fish *Pomacanthus imperator* (35), those of the zebrafish appear to be fixed to the skin. However, our perturbation experiments have revealed that the mechanism underlying the pattern formation of zebrafish is highly dynamic and autonomous. This finding narrows down the possible theoretical models that form the basis of the molecular data of the stripe pattern.

In this study, we simulated pattern formation by using an RD-based model; in this model, diffusion serves as the process of signal transfer. However, it is also possible to generate similar spatial patterns with other modes of signaling; e.g., by mechanical pressure (mechanochemical model) (46), cell movement (chemotaxic model) (47), and neuronal signaling (neural model) (48). Because these models share the same mathematical basis, which is "local self-enhancement and long-range inhibition" (6–9), it is difficult to determine the most appropriate mechanism by comparing their simulations with the macroscopic pattern changes observed. We presume that diffusion is the most likely basis of the long-range signaling in the skin; however, this assumption must be corroborated by experimental identification of the corresponding signaling molecule.

In the first experiment, we ablated all of the pigment cells in a large region to remove the original pattern. In the early stages of regeneration, the two types of pigment cells (melanophores and xanthophores) appeared in a random manner regardless of the pattern of the original stripes (Fig. 1*A*); this suggests that the precursor cells of either pigment cell type were not distributed according to any particular prepattern. Gradually, the two cell types segregated into their respective stripes, which retained their original spacing (Fig. 1 *B* and *C*). However, the orientation and position of the regenerated stripes (Fig. 1*D*) did not resemble those of the original stripes. This clearly indicates that at least until this stage, there were no other controlling signals at work; e.g., those derived from the overall gradient of some morphogen or hidden anatomical structures. Obviously, stripe formation was driven by local interactions and, as demonstrated by our simulations, displayed the typical time course of RD reactions. By using the temperature-sensitive alleles of *fms* receptor tyrosine kinase, which is expressed by xanthophores and is essential for melanophore survival and migration into stripes (25), it has been demonstrated that the haphazard stripes are regenerated when xanthophores are restored in both anal and caudal fins originally occupied only by melanophores (32). This suggests that the essentially identical mechanism is at work in the fins.

Because the RD mechanism can determine only the spacing and not the direction of the stripes (5, 49), it is reasonable that the regenerated stripe patterns lose their horizontal orientation when regeneration starts from a random pattern (Fig. 1 *A–D*). At the onset of the patterning process, RD mechanisms are highly sensitive against any local cue that induces the symmetry breaking (50). To generate the directional stripes, some additional conditions are required (e.g., directional initial distribution or directional diffusion of components) (51, 52). It is suggested that the initial organization of the melanophores, which appear along the horizontal myoseptum (19), in the juvenile fish acts as initial conformation and may enforce making the directionality of stripes.

In the second experiment, it has been shown that the stripe of zebrafish reacted to the artificially introduced partial perturbation in the stripes with the dynamics estimated by RD model (Fig. 2). According to the findings of our simulation, the dynamic rearrangement of the stripe of zebrafish occurred because, with repeated irradiation, the ability of the melanophores to recover the maximum level of activity from a low level was abolished. This was included in the simulation by eliminating the production of the baseline activator that would allow *de novo* triggering of the following reactions. Because inhibitor was not produced in the nonactivated region, the activation spread from the existing stripe into the nonactivated region, and this process led to the movement of the stripe.

The equations in the RD model usually comprise two or three diffusible substances that play multiple roles (3–8). To use theoretical models in a working hypothesis for future molecular genetic experiments, it is essential to detect what the variables in the equations represent in the real system. In the case of zebrafish, many experimental data have shown that the mutual interaction between melanophore and xanthophore plays the key role in the stripe formation (32, 37–40). In the mutant zebrafish lacking one of the two kinds of pigment cells, the remaining pigment cells form only a rudimentary pattern, whereas introduction of one pigment cell type into a mutant possessing only the other type is sufficient to make an original stripe pattern (32). From the experiments manipulating the melanophore number by making the interspecific hybrids, it is suggested that evolutionary changes in the pigment cell number have contributed to the pattern alteration (39, 40). From these experimental data, it seems reasonable to assume that the two types of pigment cells play the role of the substances in the RD model. If so, any gene mutation that changes the interaction between the pigment cells may change the pattern. This is equivalent to the change of a parametric value in the RD equation. It is notable that the allelic differences in the pigment pattern of *leopard* mutant are well reproduced by the RD simulation varying only a single parametric value (53). Recently, some mutant genes that cause the abnormal pattern have been identified (22–27). Functional analysis of the gene products will provide us the substantial reference to construct the molecular network, which may satisfy the condition of "local self-enhancement and long-range inhibition."

Materials and Methods

Fish Stock. Zebrafish were bred and maintained under standard laboratory conditions (54). The wild-type strain used was *Tü*. The age of the fish used in the experiments was ≈3 weeks after hatching, the point at which the first three stripes appear on the body.

Laser Ablation of Pigment Cells. Before ablation, the fish were anesthetized by using 0.01% MMS (ethyl-*m*-aminobenzoate methanesulfonate) and mounted on a chamber glass slide. During ablation, fish were always maintained under moist conditions with a weaker anesthesia than full anesthetization. Ablation was performed by using a 365-nm multiple light pulse laser from the MicroPoint pulse laser system (Photonic Instruments), which was focused to a ×40 objective on a microscope. In general, each pigment cell was broken down sufficiently by four to five laser pulses. Cell death from ablation was checked the next day, and remnants were ablated if there were any.

Time-Lapse Observations and Recordings. The fish used in experiments were maintained individually. Captured time-lapse images of pattern regeneration and/or striping dynamics of the ablated regions were recorded every day by using a digital camera mounted on a dissecting microscope at a fixed magnification. These recordings were continued for a duration of ≈50–60 days.

Computer Simulations. The differential equation (Gierer–Meinhardt model) (6, 8) used was

$$\frac{du}{dt} = a - bu + \frac{u^2}{v(1 + cu^2)} + D_u \Delta u$$

$$\frac{dv}{dt} = u^2 - v + D_v \Delta v,$$

where u and v are the concentrations of hypothetical factors. u and v act as the activator and the inhibitor, respectively. Parameters were $a = 0, b = 1.2, c = 0.4, D_u = 1 \times 10^{-2}$, and $D_v = 20 \times 10^{-2}$. To adjust the parameters so as to form the stripes, it is crucial that the saturation level of the self-enhancement of activator (parameter c) is adjusted appropriately (8). We set as $c = 0.4$. With smaller or larger value of c, the resulting pattern becomes spots or network.

Simulations in the both first (Fig. 1 *E–H*) and second (Fig. 2 *E–H*) experiment, a parallel pattern was set as an initial condition. After this, both substances in the square area were removed and replaced by the random pattern of substance u (mean = 0.5, SD = 1) in the first experiment (Fig. 1*E*). The field size was 256×128. On the other hand, in the second experiment, both substances in the square region were continuously removed (Fig. 2 *E–G*). When the calculation began, the terminated ends of the cut stripes elongated to fill the vacant space. To avoid this effect, the end of elongating stripes was manually erased. The field size was 80×80.

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