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Review

Unifying principles of regeneration I: Epimorphosis versus morphallaxis

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Because research on regeneration has a long history, some classic definitions and concepts about regeneration which were established in earlier times have been retained without reconsideration for a long time, even though many relevant new findings have accumulated. To clarify the points on which research should be focused on for elucidating the mechanisms of regeneration, we should reconsider such classical definitions and principles of regeneration at the cellular and molecular level. Here, we consider two differing principles of regeneration which have been classically defined as 'epimorphosis' and 'morphallaxis', and propose the abandonment of these classical categories and their replacement by a new unifying principle in order to facilitate regeneration studies.

Key words: epimorphosis, intercalation, morphallaxis, planarian, regeneration.

Introduction: Epimorphosis versus morphallaxis

As students, we learned that regeneration can be divided into two types according to its mode; either epimorphosis or morphallaxis (Fig. 1). In the case of newt limb regeneration, the residual part of the limb remains as it is and a 'blastema' forms at the site of the wound and eventually regenerates the lost tissues and organs (Fig. 1A). This kind of 'add-on' regeneration is called 'epimorphic regeneration' (Suzuki *et al.* 2006). In this type of regeneration, the old stump provides cells participating in blastema formation without drastic rearrangement of the remaining tissues. In contrast, in the other type of regeneration, for example in hydra regeneration, a blastema is not formed on the wound surface; rather, the remaining part is drastically remodeled to regenerate all parts of the body (Fig. 1B). This type of remodeling regeneration is called morphallaxis. Simply speaking, regeneration can be classified as epimorphic or morphallactic according to whether or not a blastema is formed after wound healing.

The definition of a blastema is slightly vague, but a blastema can easily be recognized as a 'white region' formed on the cut surface of the body. Some factor(s) maintaining the undifferentiated state of cells in the blastema may also inhibit the differentiation of pigment cells. This may be the reason why the blastema can be recognized as a white area during regeneration, but nobody has succeeded in identifying such factor(s). If we examine this white region by making histological sections, we can easily confirm that the blastema is actually composed of typical morphologically undifferentiated cells.

What type of regeneration occurs in the case of planarian regeneration? When we cut planarians, a typical blastema is formed, which can be recognized as a white region by the naked eye and as a mass of undifferentiated cells by histological observation (Reddien & Sánchez-Alvarado 2004). However, a pioneer in planarian regeneration, Thomas H. Morgan, classified planarian regeneration as morphallaxis about 100 years ago, although he had observed blastema formation (Morgan 1900). In contrast, Takashi Kido in Japan carefully observed pharynx regeneration in the tail pieces and concluded that although a new pharynx was formed in the central portion of the

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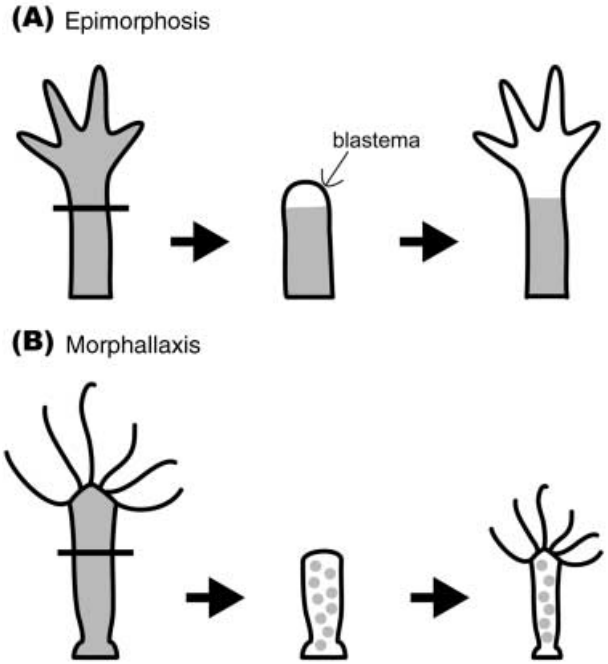

Fig. 1. Typical classical examples of epimorphic and morphallactic regeneration. (A) Limb regeneration in amphibians is a representative example of epimorphosis. In this type of regeneration, a mass of undifferentiated cells referred to as the 'blastema' is initially formed after wound healing and then blastema cells actively proliferate to restore the lost part of the amputated organ. (B) Hydra regeneration is categorized as morphallaxis. A blastema is not formed. Direct rearrangement of pre-existing cells in the stump contributes to regeneration.

stump, the cells participating in pharynx formation appeared to be derived from the blastema and to migrate to the posterior region (Kido 1959, 1961), supporting the notion that regeneration in planarians is epimorphic. As a result, some biology textbooks used in Japanese high schools described planarian regeneration as epimorphosis.

Is planarian regeneration actually epimorphosis or morphallaxis? To answer this question, we carefully observed planarian regeneration at the cellular level using specific molecular markers (Umesono *et al*. 1997; Agata *et al*. 1998; Kobayashi *et al*. 1998; Shibata *et al*. 1999; Cebrià *et al*. 2002b) and a fluorescence activated cell sorter (Ogawa *et al*. 2002a; Hayashi *et al*. 2006) and found that we must change the old definitions concerning regeneration. The old definitions were made without observations at the cellular level and this caused some confusion in the field of regenerative biology. We propose that all regeneration phenomena should be reinvestigated at the cellular level and that the classical categories of regeneration must be reconsidered.

Intercalary regeneration in planarians

When we started our observation of planarian regeneration 15 years ago, we believed that planarian regeneration was a typical example of epimorphic regeneration. At that time, we generally observed the regeneration process from trunk pieces, because we could simultaneously see the process of regeneration of both the head and tail regions from the trunk pieces. As long as we were observing the regeneration process from the trunk pieces, we did not doubt that planarians underwent epimorphic regeneration, because lost tissues were formed from the blastema. However, when we carefully observed the process of regeneration from the tail pieces, we noted that the pharynx was formed in the central portion of the remaining tail fragments, leading us to think that pharynx regeneration resulted instead from morphallaxis. Morgan had described the formation of a regenerating pharynx in the remaining part of the body (he called 'old tissue') when he observed its regeneration from small pieces (Morgan 1898) and classified planarian regeneration as morphallaxis (Morgan 1900). However, while trying to interpret our findings, we found Kido's papers (Kido 1959, 1961) and realized that he had reached the same conclusion as us although he had interpreted this as epimorphic regeneration of the pharynx. That is, he found that the cells in the anterior blastema seemed to migrate posteriorly and to participate in epimorphic regeneration of the pharynx.

To determine if planarian regeneration occurs via epimorphosis or morphallaxis, we isolated a pharynx muscle-specific myosin heavy chain gene (*DjMHC-A*; Kobayashi *et al*. 1998) and followed cell commitment and migration using a *DjMHC-A* RNA probe (Kobayashi *et al*. 1999a). Interestingly, we obtained unexpected results. *DjMHC-A*-positive cells were detected in the central portion of the tail fragments, the locale of the future pharynx-forming region, but not in the blastema, suggesting that stem cells may be committed in the mesenchymal space of the tail fragment prior to their migration into the pharynx rudiment. A similar result was obtained when we observed the pharynx regeneration from the head piece. *DjMHC-A* mRNA-positive cells appeared in the mesenchymal space in the old stump region juxtaposed to the blastema (Kobayashi *et al*. 1999a).

We also traced the formation of mucus-producing cells during regeneration using a cell type-specific RNA probe and obtained similar results, indicating that stem cells located in the mesenchymal space were committed in a position-dependent manner prior to their migration to the final target positions (Agata & Watanabe 1999; Agata *et al.* 2003). No positive cells could be

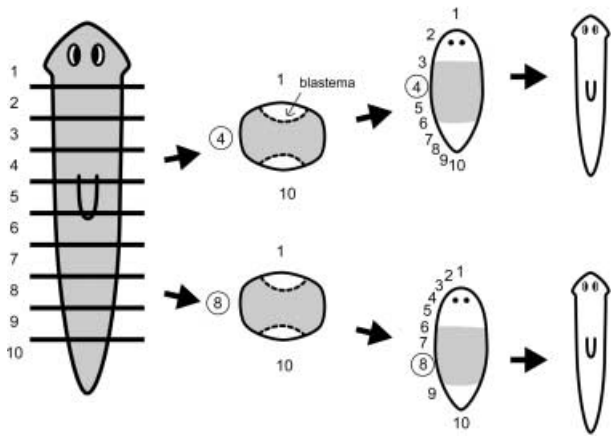

Fig. 2. Schema of intercalary regeneration in planarians after amputation into 10 pieces. Observation of the regeneration processes of the level 4 and 8 pieces reveals that the anterior and posterior blastema always have positional values 1 and 10, respectively, even though their original stumps have different original values. Then, the newly formed blastema interacts with the old stumps to induce intercalary events. Finally, stem cells located in the mesenchymal space start to differentiate appropriate cell types according to the newly acquired positional values.

detected in the blastema. We then found that a brain was always generated from the anterior blastema even though we cut planarians at different positions of the bodies along the anteroposterior (A–P) axis, whereas the posterior blastema always generated a tail region. From these observations we proposed an 'intercalary model' of planarian regeneration (Fig. 2; Agata *et al*. 2003). In this new model, the anterior and posterior blastemas were formed as signaling centers to direct intercalary reorganization of body regionality or positional information. After rearrangement of the body regionality, stem cells located in the mesenchymal space may become committed to differentiate to appropriate cell types depending on the newly acquired positional information. We demonstrated by grafting experiments that intercalary regeneration does in fact occur in planarians (Kobayashi *et al*. 1999b). We also demonstrated that such intercalary regeneration can be seen along both the dorsoventral and mediolateral axes (Kato *et al*. 1999; Saito *et al*. 2003).

Such cellular-level observations clearly indicated that planarian regeneration cannot be classified into epimorphosis or morphallaxis, even though a blastema is formed at the cut surface. Rather, in the case of planarians, a blastema is formed as a signaling center to reorganize body regionality.

Reconsideration of epimorphosis

Limb regeneration of amphibians is one of the most extensively studied cases in the field of regenerative biology (Nye *et al*. 2003; Suzuki *et al*. 2006). A great deal of knowledge has been accumulated on this phenomenon and the word blastema was made based on observations of limb regeneration. When one observes the early steps of limb regeneration by making paraffin sections, it can be seen that the white region which is formed on the cut surface is composed of a very homogeneous cell population showing undifferentiated morphology. A mass of these cells appears to be formed in the distal part of regenerating limbs. In contrast, in the old stump, many hard tissues, including bones, remain intact. Thus, people who observed limb regeneration generally believed that limb regeneration occurred via epimorphosis. The word epimorphosis means add-on regeneration of proximal parts to distal parts without affecting the remaining tissues. However, we need to reconsider if it is still appropriate to use such a word to describe limb regeneration.

It has been demonstrated that the distal part is first formed and then proximal parts are formed by intercalation during limb regeneration (Iten & Bryant 1975; Torok *et al*. 1998). In fact, an 'intercalation model' has been developed based on the results of grafting experiments of limbs of urodeles and cockroaches (Iten & Bryant 1975; French *et al*. 1976). The early editions of Scott Gilbert's textbook *Developmental Biology* explained intercalary regeneration using amphibian limb regeneration as a model. Recently, a review of intercalary regeneration of limb regeneration was published by David L. Stocum's group in *Developmental Dynamics* (Nye *et al*. 2003). These authors recognized that rearrangement of positional information by intercalary events may occur not only in morphallactic regeneration but also in epimorphic regeneration.

Susan V. Bryant and David M. Gardiner divided limb regeneration into three phases: wound healing, dedifferentiation and redevelopment (Bryant *et al*. 2002; Gardiner *et al*. 2002), with the redevelopment phase completely mimicking embryonic development. Thus, after blastema formation, the same genetic program used in development may work to regenerate the original structure. However, several important differences from normal development have been reported in the dedifferentiation phase. For example, the expression patterns of Hox genes are somewhat different between them. Hox genes are believed to be involved in pattern formation in early steps of both development and regeneration. Re-expression of the Hox genes is initiated at a very early stage of regeneration in internal tissues of the stump prior to blastema formation (Gardiner *et al*. 1995; Torok *et al*. 1998). Interestingly, these genes are initially expressed in the same

population of stump cells and then the expression domains of these genes after blastema formation become spatially distinct as growth progresses. These results clearly indicate that reorganization of positional information occurs in the stump region immediately after wound healing prior to blastema formation. We speculate that the blastema is formed as a result of intercalary events at the early stage of limb regeneration.

Unifying the principles of regeneration

Here we have introduced several different cases of regeneration. Some of them have in the past been classified as morphallaxis and some as epimorphosis, while some of them could not be classified into either model. However, such categorization leads to a misunderstanding of regeneration, suggesting that regeneration in different animals may be controlled by different principles. However, regeneration is always conducted under the control of positional information. The distal portion of the body is formed immediately after wound healing around the cut surface (a step called 'distalization'), and interaction of the newly formed distal portion and the remaining proximal portion may induce reorganization of positional information and lost tissues are then intercalatively generated to restore the original structures ('intercalation'). In our opinion, this is probably a general principal of regeneration from invertebrates to vertebrates (Fig. 3). However, the tissues acquiring distal characteristics and cells participating in the regeneration of lost tissues vary among different animals and different regeneration systems.

For instance, in the case of limb regeneration, the epidermis covering the wound surface may acquire the distal character to induce intercalation, and then the blastema is formed as the result of intercalation between the wound epidermis (distal identity) and the remaining part of the body (proximal identity) and actively proliferates to restore lost tissues and organs (Nye *et al.* 2003). However, in the case of planarian regeneration, we could not detect any proliferating cells in the blastema. In planarians, the stem cells participating in blastema formation may stop proliferating and acquire a distal character to induce intercalary events. Stem cells located in the mesenchymal space of the old stump then start to differentiate into appropriate cell types depending on the newly acquired positional information (Agata *et al.* 2003). Thus, the blastema of planarians may work as a signaling center rather than as the place forming lost tissues and organs. In hydra, a blastema is not formed, probably because the differentiation state of all of the cells, including neurons, may be highly plastic (Koizumi & Bode 1991). When ectodermal and endodermal cells covering the wound surface in hydra acquire distal characteristics and start to induce reorganization of the body regionality by intercalation, differentiated cells, including neurons, change their phenotype according to the newly acquired body regionality. Interstitial cells (multipotent stem cells) located between the ectodermal and endodermal layers also change their differentiation patterns according to the newly acquired body regionality, resulting in complete restoration of all parts of the body.

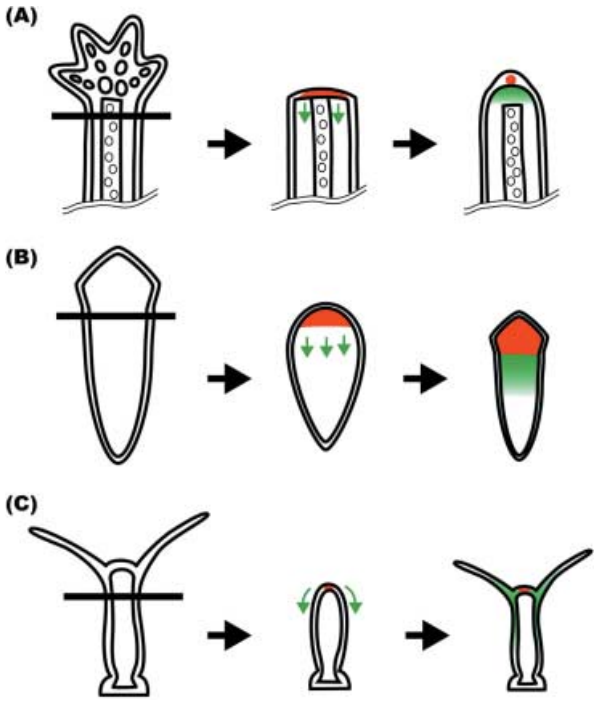

Fig. 3. Comparison of three different cases (A, urodele's limb; B, planarian; C, hydra) of regeneration from the new viewpoint (distalization followed by intercalation) proposed in the present review. In all cases, the most distal portion is formed immediately after amputation (indicated in red) and then intercalary events induce the formation of the intermediate region between the newly formed distal portion and the old proximal portion (green arrows). However, the cell types or tissue layers participating in distalization and intercalation vary among the different cases.

Based on these observations, we propose that 'epimorphosis' and 'morphallaxis' should no longer be used to classify regeneration phenomena, because regeneration cannot actually be divided into these categories. The more useful concepts of 'distalization' and 'intercalation' should instead be used to explain the principles of regeneration. Recent regeneration studies using crickets' legs also support our proposal (Mito *et al.* 2002; Nakamura *et al.* 2007).

Is there any molecular commonality in regeneration?

To restore the original structure and function, the most important molecular event is the reorganization of positional information. Here, we propose that 'distalization' and 'intercalation' may be general principles underlying regeneration. To regenerate all of the lost body parts, organisms initially form the most distal part and then reconstitute the intermediate region by appropriate intercalation of newly generated tissues between the newly formed distal part and the remaining body part. Is there any commonality in the molecular mechanisms underlying regeneration in various organisms?

We suppose that there is no clear commonality of the molecules regulating distalization and intercalation among various organisms, but that there is some commonality of the molecular systems realizing the reorganization of positional information. For example, in the case of planarians, we demonstrated that dorsoventral intercalation may be mediated by the interaction of bone morphogenetic protein (BMP) and its antagonist, Djnlg (the product of *Dugesia japonica*'s *noggin*-like gene) (Orii *et al*. 1998; Ogawa *et al*. 2002b). This molecular interaction may also induce both blastema formation and distalization. Thereafter, in the anterior blastema, the signaling of fibroblast growth factor (FGF) and its neutralizing receptor '*nou-darake*' is specifically activated to form a brain in the head region (Cebrià *et al*. 2002a). Such molecular interactions between morphogens and their antagonists/neutralizing receptors are commonly and widely observed to be involved in the production of positional information. In the case of hydra regeneration, Wnt signal-related genes are specifically activated immediately after amputation of the anterior portion (Hobmayer *et al*. 2000), and the interaction of Wnt and its antagonist dickkopf may then reorganize body patterning along the A–P axis (Augustin *et al*. 2006; Guder *et al*. 2006). In the case of leg regeneration of crickets, the formation of the proximodistal axis of a regenerating leg is triggered at a site where ventral *wg*-expressing cells abut dorsal *dpp*-expressing cells in the A–P boundary, as postulated in the boundary model modified by Campbell and Tomlinson (Campbell & Tomlinson 1998; Mito *et al*. 2002; Nakamura *et al*. 2007). In the case of limb regeneration of amphibians, it has been reported that FGF signaling may have an important role in the stimulation of regeneration (Christen & Slack 1997; Yokoyama *et al*. 2001). It has also been demonstrated that intercalary regeneration of a *Xenopus* limb was induced by implantation of an FGF8-soaked bead at stages 52 and 53 (Shimizu-Nishikawa *et al*. 2003). However, the involvement of molecule(s) antagonizing FGF signaling has not yet been reported in limb regeneration.

Recently, Reversade and De Robertis (2005) have succeeded in elucidating the molecular mechanisms underlying the self-regulation of the complete regeneration of early *Xenopus* half-embryos. They demonstrated that dorsal (ADMP) and ventral BMP (BMP2/4/7) signals and their extracellular antagonists expressed under opposing transcriptional regulation (Chordin and Sizzled) provide a molecular mechanism for embryonic self-regulation. This model is fascinating and similar types of molecular systems may be involved in distalization and intercalation during regeneration.

Conclusion

Regeneration phenomena have been extensively studied in order to understand how organisms self-organize their complicated body structures. However, the cellular and molecular mechanisms underlying regeneration remained unclear for a long time. We speculate that one of the reasons progress was so slow was the misunderstanding of certain principles of regeneration. In this review we proposed doing away with the classical categorization of regeneration as either 'epimorphosis' or 'morphallaxis'. We emphasize instead that investigating how positional identity is rearranged after amputation is the most important point for understanding regeneration, and that we should investigate what type of cells contribute to reorganization of body regionality and what type of cells participate in the regeneration of lost tissues at the cellular level. Here, we proposed reconsideration of different types of regeneration using 'distalization' and 'intercalation' as key concepts, and reviewed molecular mechanisms underlying regeneration in terms of these key concepts. In the next review, we will discuss the cell types participating in regeneration after the reorganization of body regionality.

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