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Planarians Maintain a Constant Ratio of Different Cell Types During Changes in Body Size by Using the Stem Cell System

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Planarians change in body size depending upon whether they are in feeding or starving conditions. To investigate how planarians regulate this flexible system, the numbers of total cells and specific cell types were counted and compared among worms 2 mm to 9 mm in body length. The total cell number increased linearly with increasing body length, but the ratio of cell numbers between the head and the trunk portion was constant (1:3). Interestingly, counting the numbers of specific neurons in the eye and brain after immunostaining using cell type-specific antibodies revealed that the ratio between different neuron types was constant regardless of the brain and body size. These results suggest that planarians can maintain proportionality while changing their body size by maintaining a constant ratio of different cell types. To understand this system and reveal how planarians restore the original ratio during eye and brain regeneration, the numbers of specialized cells were investigated during regeneration. The results further substantiate the existence of some form of "counting mechanism" that has the ability to regulate both the absolute and relative numbers of different cell types in complex organs such as the brain during cell turnover, starvation, and regeneration.

Key words: planarian, regeneration, stem cell, body proportion, brain, growth, degrowth

INTRODUCTION

The mechanisms regulating body proportions in multicellular organisms remain largely unknown. In human beings, the ratio between the length of the head and the rest of the body generally ranges from 1:5 to 1:7 among different populations and individuals. Studies on *Drosophila* have shown that the insulin signaling or Fat signaling pathway controls the size of cells, organs, and bodies (Hafen and Stocker, 2003; Cho et al., 2008). However, in addition to body segment proportions, the size proportions among different organs inside bodies are maintained by yet unknown mechanisms. Can different organs communicate with each other to determine their size? Do any organs have the capacity to determine the size of other organs?

Planarians are uniquely useful animals for investigating mechanisms involved in maintaining body proportions and in maintaining the sizes of various organs during development and after maturation. Planarians can change in body size depending upon feeding conditions, and can also restore the original proportions during regeneration, and degrow under starvation conditions instead of dying. For instance, if planarians are not fed anything for 3 months, worms about 10 mm in length degrow to about 1 mm. When planarians 10 mm long are fed, they start self-fission after reaching about 12 mm in body length at 23°C. To begin to investigate these phenomena, we counted total cell numbers in worms of various sizes. We investigated the head-to-tail proportion among worms of various sizes by comparing cell numbers between the head and trunk fragments in decapitated animals.

Previous anatomical studies quantified various cell types by disrupting degrowing and regenerating animals, and showed that the total cell number increases linearly with body size; that the relative proportion of different cell types is largely constant in animals of different size and is not substantially changed by starvation; and that degrowth changes the body length by changing the overall cell number, rather than by changing cell size (Baguñá and Romero, 1981; Romero and Baguñá, 1991). However, these analyses were conducted through morphological observations after maceration. A previous planarian study using molecular markers showed that the number of cells expressing the marker gene *cintillo*, which shares homology with the DEG/ENaC superfamily of sodium channels, changes depending on the body size (Oviedo et al., 2003). To provide a high degree of resolution, in this study, we quantitatively investigated not just a single type of cell but various types of cells by whole-mount staining using molecular markers and determined the relationship between the numbers of these cells in the whole body and in some organs. In particular, we analyzed eyes and brains in order to compare the size and cell populations of these specific organs relative to body size.

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Dugesia japonica has a pair of eyes and a brain in the head. The brain is composed of two lobes with nine lateral branches and forms an inverted U-shaped structure (Agata et al., 1998; Nakazawa et al., 2003; Okamoto et al., 2005; Tazaki et al., 1999). Previous studies showed that the planarian brain is composed of several functionally different domains distinguished by discrete expression of three *Otx*/*otp*-related genes (Umesono et al., 1997; 1999). In addition, recent studies revealed that the planarian brain consists of various types of neurons synthesizing different neurotransmitters, including GABAergic, dopaminergic, octopaminergic, and serotonergic neurons, and that these neurons are differently distributed in the brain (Cebrià, 2008; Nishimura et al., 2007a, b; 2008a, b). A pair of eyes located on the dorsal side of the head is composed of two types of cells: visual neurons and pigment cells. The visual neurons are photosensory and project axons to the brain. The pigment cells contain pigment granules and form the black eye cup (Asano et al., 1998; Inoue et al., 2004). Our laboratory has developed antibodies to detect GABAergic, dopaminergic, octopaminergic, and serotonergic neurons in the brain, as well as visual neurons and pigment cells in the eye (Nishimura et al., 2007b; Sakai et al., 2000). By using these antibodies to count the number of each type of neuron, we investigated whether the number of neurons changes in planarians of different sizes, and whether the ratios between the number neurons of each type and the numbers of cells of other cell types are strictly regulated.

Planarians have high regenerative ability. Even when they are cut into very small pieces, most pieces can regenerate a whole body within 7 days. Previous studies have shown that during regeneration, planarians regenerate a functional brain and eyes from pieces not initially containing these tissues or their constituent cell types (Agata and

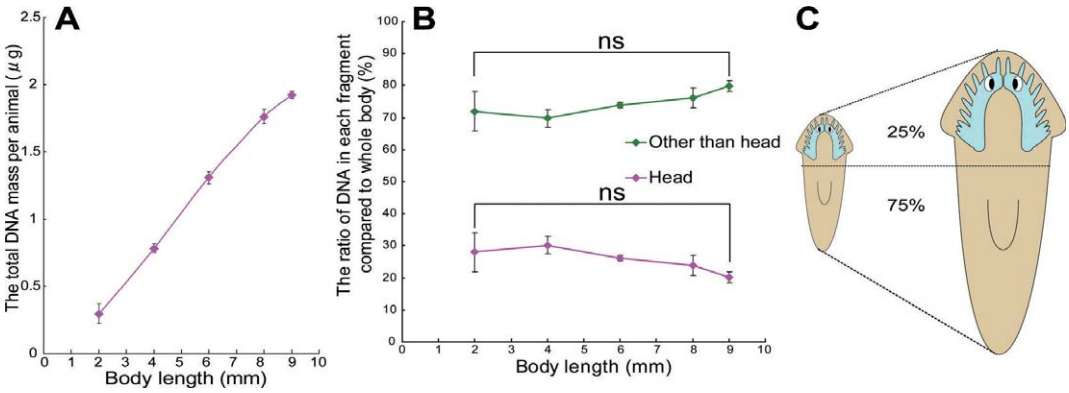

Fig. 1. Planarian head proportion relative to the whole body. **(A)** Relationship between total DNA mass, total number of cells, and body length. **(B)** Ratio of DNA in head and tail fragments relative to the whole body. Planarians maintain the head proportion relative to other body parts regardless of body length. **(C)** Schematic drawing of planarian growth. Error bars, S.E.M.; ns, not significant (P>0.05); n>3 in each experiment.

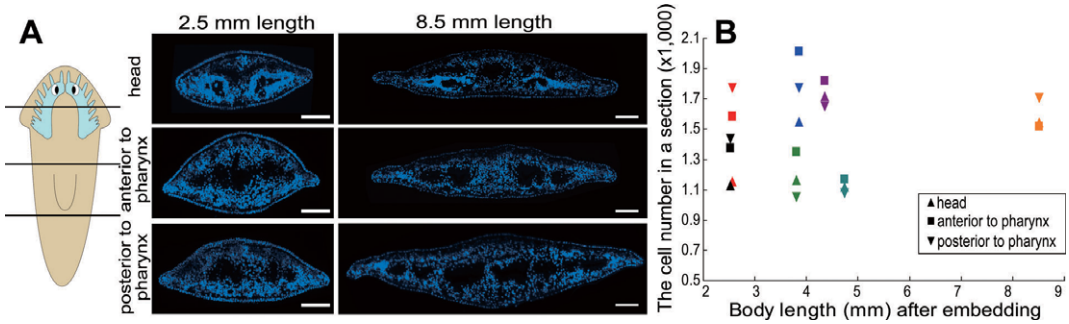

Fig. 2. Number of cells per transverse section in different-sized planarians. **(A)** Transverse sections at three different points in planarians 2.5 and 8.5 mm long. The number of cells detected per transverse section was counted after staining with Hoechst 33342. The shapes of the transverse sections were deformed during fixation. Scale bars: 100 μm. **(B)** Number of cells per section. Points in the same color represent data from the same individual planarian.

Table 1. Antibodies used in this study.

antigen	cell type	animal	reference
Djsyt (*D.japonica* synaptotagmin)	pan neural	mouse (polyclonal)	Tazaki et al. (1999)
Djarrestin (*D.japonica* arrestin)	visual neuron	rabbit (polyclonal)	Sakai et al. (2000)
DjTPH (*D.japonica* tryptophan hydroxylase)	pigment cell serotonergic neuron	mouse (monoclonal)	Nishimura et al. (2007b)
DjGAD (*D. japonica* glutamic acid decarboxylase)	GABAergic neuron	mouse (monoclonal)	Nishimura et al. (2008a)
DjTH (*D. japonica* tyrosine hydroxylase)	dopaminergic neuron	mouse (monoclonal)	Nishimura et al. (2007a)
DjTBH (*D. japonica* tyramine β-hydroxylase)	octopaminergic neuron	rabbit (polyclonal)	Nishimura et al. (2008b)

Umesono, 2008; Inoue et al., 2004). We also counted the number of these constituent cells during regeneration, and then investigated the mechanisms underlying the maintenance and restoration of body proportions.

MATERIALS AND METHODS

Animals

A clonal strain of planarian (*Dugesia japonica*), SSP, was used in all experiments (Ito et al., 2001). The planarians were cultured at 23°C in autoclaved tap water and starved for at least 1 week before any experimental procedure.

Whole-mount immunostaining

Planarians were treated with 2% HCl in 5/8 Holtfreter's solution for 10 min before fixation. Animals were then fixed in either a paraformaldehyde solution or Carnoy's solution, depending on the primary antibody to be used. For the anti-DjTH mouse monoclonal and anti-DjTBH rabbit polyclonal antibodies, planarians were fixed in 4% paraformaldehyde containg 5% methanol for 3 h at 4°C (Takano et al., 2007). For the anti-Djarrestin rabbit polyclonal, anti-DjTPH mouse monoclonal, and anti-DjGAD mouse monoclonal antibodies, planarians were fixed in Carnoy's solution (ethanol:chloroform:acetic acid anhydride in a proportion of 6:3:1) for 3 h at 4°C (Umesono et al., 1997). After fixation, samples were bleached with 5% H_2O_2 in PBST (phosphate-buffered saline containing 0.1% Triton X) for 16 h at room temperature under fluorescent light. After bleaching, planarians were blocked against nonspecific binding with 10% goat serum in PBST for 2 h. Planarians were then treated overnight at 4°C with specific primary antibodies in PBST containing 10% goat serum. We used the anti-Djarrestin rabbit polyclonal antibody (at 1:1000 dilution) to detect photoreceptor neurons; the anti-DjGAD and anti-DjTPH mouse monoclonal antibodies to visualize GABAergic neurons and pigment cells, respectively; the anti-DjTBH rabbit polyclonal antibody (at 1:1000 dilution) to visualize octopaminergic neurons; and the anti-DjTH mouse monoclonal antibody (at 1:5000 dilution) to visualize dopaminergic neurons. Samples were then treated overnight at 4°C with a secondary antibody conjugated with Alexa Fluor 488 or 594 (Molecular Probes), at a dilution of 1:500 in PBST containing 1 μg/ml Hoechst 33342 (Invitrogen) for nuclear staining. A BX62 microscope (Olympus) was used to detect fluorescence.

Immunostaining of sections

Planarians were fixed with a modified relaxant solution (1% HNO_3, 2.25% formalin, 0.05 mM $MgSO_4$ in 5/8 Holtfreter's solution) overnight at 4°C (Dower, 1973; Kobayashi et al., 1998). After fixation, animals were bleached with 5% H_2O_2 in PBST for 16 h at room temperature under fluorescent light. Bleached samples were dehydrated with ethanol solutions of gradually increasing concentration and transferred to xylene. The dehydrated samples were then embedded in paraffin and sectioned at 8–10 μm. Sections were deparaffinized with xylene, rehydrated

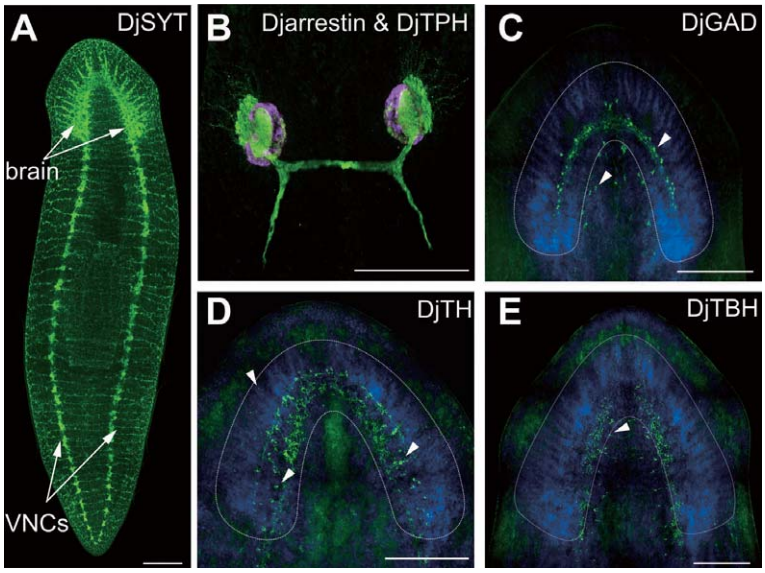

Fig. 3. Planarian CNS and eye. **(A)** Planarian CNS stained with the anti-DjSYT antibody. **(B)** Double immunostaining of planarian eyes using the anti-DjTPH (magenta) and -Djarrestin (green) antibodies, which stain pigment cells and visual neurons, respectively. **(C–E)** Localizations of various types of neurons. GABAergic, dopaminergic, and octopaminergic neurons were stained with the anti-DjGAD, -DjTH, and -DjTBH antibodies, respectively. Arrowheads indicate some neurons of each type. Blue indicates nuclei stained with Hoechst 33342. Broken lines outline the brain. Scale bars: 300 μm.

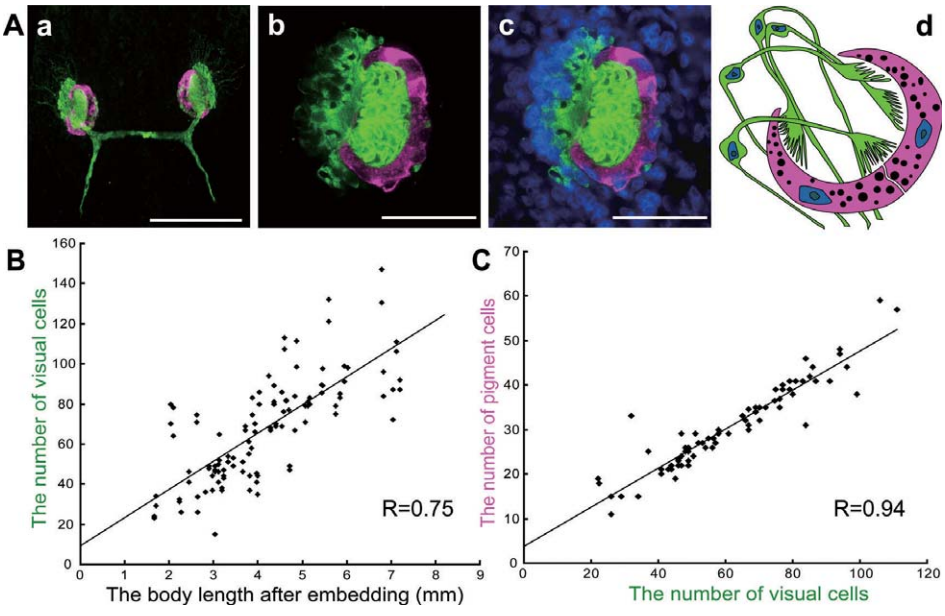

Fig. 4. Structure of the intact planarian eye. **(A)** Double immunostaining with anti-DjTPH (magenta) and -Djarrestin (green). **(A-b, c)** Magnified views of the left eye. Blue indicates nuclei stained with Hoechst 33342 (A-c). **(A-d)** Diagram of the planarian eye. Scale bars: 300 μm (A-a), 50 μm (A-b and c). Each eye is composed of two types of cells: visual neurons (green) and pigment cells (magenta). **(B)** Correlation between planarian body length after embedding and the number of visual neurons. $P<0.001$. **(C)** Graph showing the correlation between the numbers of visual neurons and pigment cells in intact animals. Each point represents an individual planarian. $P<0.001$.

in a series of ethanol solutions of gradually decreasing concentration, and blocked against nonspecific binding with 10% goat serum in PBST for 2 h. Sections were then treated overnight at 4°C with the primary antibody, the anti-Djarrestin rabbit polyclonal antibody or anti-DjTPH mouse monoclonal antibody, in PBST containing 10% goat serum. Sections were then treated with a secondary antibody conjugated with Alexa Fluor 488 or 594 (Molecular Probes), used at a dilution of 1:1000 in PBST. After washing, samples were treated with 1 μg/ml Hoechst 33342 (Invitrogen) for 10 min for nuclear staining.

In-situ hybridization of sections

Samples were sectioned by using a method similar to that used for immunostaining. A biotin-labeled antisense RNA probe and a digoxigenin (DIG)-labeled antisense RNA probe were prepared by using *Djarrestin* and *DjTPH* cDNA as templates, respectively. Sections were deparaffinized with xylene and ethanol solutions of gradually decreasing concentration and acetylated with acetic acid anhydride. They were then hybridized with the RNA probe, as previously described (Agata et al., 1998; Umesono et al., 1997). In-situ signals were detected by using TSA Kit 2 or 15 (Molecular Probes). Cell nuclei were labeled with Hoechst 33342. Sections were observed under a BX62 fluorescence microscope (Olympus).

Measurement of total DNA concentration

Planarians were treated first with 2% HCl in 5/8 Holtfreter's solution for 10 min and then with proteinase K (QIAGEN) overnight at 56°C according to the protocol for Biorobot M48 (QIAGEN). Planarian DNA was extracted with 200 μl of RNase-free water using BioRobot M48 (QIAGEN), and the absorbance (A260) was measured with a spectrophotometer (NanoDrop ND-1000; BMS) and used to calculate the total amount of DNA.

RESULTS

The planarian head is proportional to the whole body

To investigate whether the total cell number is proportional to body length, the total DNA was extracted from planarians of various sizes, and the total mass of the DNA was measured to estimate the number of cells (Fig. 1A). The result was that the number of cells in planarians increases with increasing body length.

Next, to investigate whether the proportion of head size relative to the whole body size is constant, planarians were cut in the region posterior to the brain, the total DNA was extracted from both the head and tail fragments, and the masses of DNA were measured (Fig. 1B). The result was that planarians have a constant ratio of head to whole body length at the cellular level, regardless of body length (from 2 to 9 mm), and that the ratio of cell number between the head and the rest of the body remains constant (1:3) (Fig. 1C).

To investigate why the total number of planarian cells changes linearly with body length, we sectioned different-sized planarians to produce transverse sections 10 μm thick and counted the cell number in sections from three points: the head, anterior to the pharynx, and posterior to the pharynx (Fig. 2). The result was that the number of cells in each section from the same point in the body did not vary, regardless of body length. Moreover, the size of most of the cells did not change drastically (data not shown). These results suggest that the number of cells in planarians increases mainly as a function of body length, and that the space inside

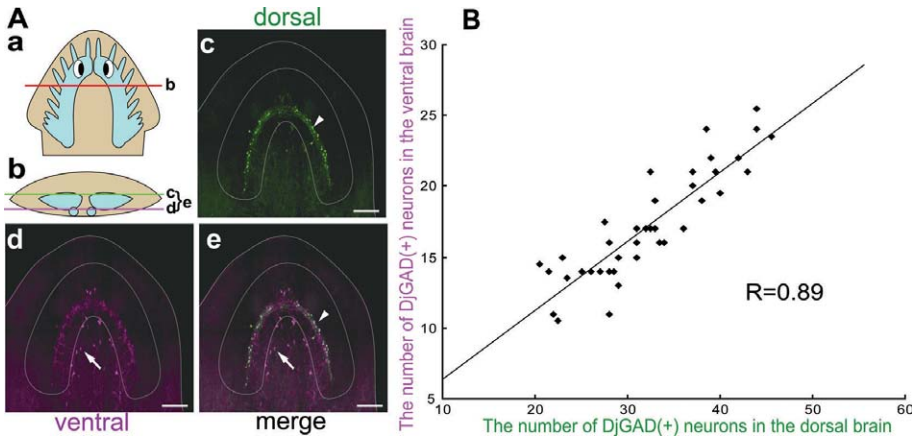

Fig. 5. Distribution pattern of DjGAD-immunopositive cells in the intact planarian head. **(A-a)** Diagram of the planarian head in dorsal view. **(A-b)** Schematic cross section of the planarian head. **(A-c, d)** Localization of DjGAD-immunopositive neurons in the dorsal and ventral parts of a brain stained with the anti-DjGAD antibody. **(A-e)** The different focal planes were pseudcolored differently, indicating that these two types of neurons are not co-localized with each other. Scale bars: 150 μm. **(B)** Correlation between the numbers of dorsal and ventral DjGAD-immunopositive neurons in intact animals. Each point represents an individual planarian. P<0.001.

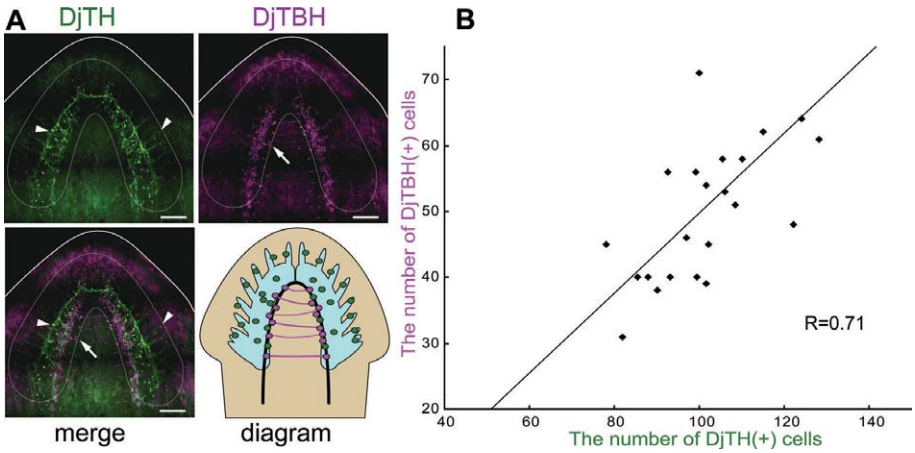

Fig. 6. Distribution pattern of DjTH- and DjTBH-immunopositive cells in the intact planarian head. **(A)** Localization of dopaminergic and octopaminergic neurons stained with the anti-DjTH and -DjTBH antibodies, respectively. Arrowheads indicate some dopaminergic neurons and arrows indicate some octopaminergic neurons. Scale bars: 150 μm. **(B)** Correlation between the numbers of DjTH- and DjTBH-immunopositive neurons in intact animals. Each point represents an individual planarian. P<0.001.

the intestinal ducts and mesenchymal tissue increases as the length increases. In this experiment, the planarian body length shrank to half due to dehydration, because it was measured after embedding in paraffin, but this did not affect the conclusions.

From the average number of cells per section and the total number of sections per animal, we estimated that a planarian with a body 6 mm long contains approximately 6×10^5 cells.

Cell proportions in the intact planarian eye

After showing that planarians maintain the proportion between the head and the whole body length, we then asked whether they maintain this proportion at the cellular level. Planarians have a CNS and one pair of eyes, and these structures are easily detected at the level of single cells by using cell-type-specific antibodies, as listed in Table 1 (see also Fig. 3). We first investigated the intact eye, which is easily recognizable. Eyes of *Dugesia japonica* are composed of two types of cells, visual neurons and pigment cells, which can be detected at the cellular level by using the anti-Djarrestin and DjTPH antibodies, respectively (Table 1; Fig. 4A). Using the antibodies, we counted these cells in the planarian eye. A statistical analysis using Pearson's product-moment correlation coefficient revealed that the number of visual neurons was correlated with body length, suggesting that planarians change the number of visual neurons according to changes in body size (Fig. 4B). Plotting the number of visual neurons versus the number of pigment cells (Fig. 4C) revealed a strong correlation between these two types of cells. These experiments showed that the numbers of both types of cells in the eye are proportional to body length, and additionally that the ratio (2:1) between the number of visual neurons and pigment cells remains constant.

Proportions of specific cell types in the brain

We next focused on another organ, the brain, at the cellular level. As described above, the planarian brain contains several types of neurons, one of which is the GABAergic neuron. GABA (γ-aminobutyric acid) is synthesized from glutamic acid by glutamic acid decarboxylase (GAD). A GAD gene, *DjGAD*, was previously isolated from the planarian *Dugesia japonica* (Nishimura et al., 2008a). DjGAD-immunopositive neurons are distributed in the most dorsal and the ventro-medial regions of the head (Fig. 5A), and GABAergic neurons play a key role(s) in the planarian photoreceptive system (Nishimura et al., 2008a). We counted and plotted the number of DjGAD (+) neurons in intact animals (Fig. 5B). There was a statistically significant correlation between the number of DjGAD (+) neurons in the dorsal brain and that in the ventro-medial region of the brain. The ratio of the number of dorsal DjGAD (+) to ventro-medial DjGAD (+) neurons was 2:1 in intact animals. This result suggests that the planarian brain maintains a constant proportion of neurons of a particular type, and expressing the same gene, between different regions.

We then investigated at the cellular level the proportion of two types of neurons synthesizing different neurotransmitters in the brain. The planarian brain contains dopaminergic neurons and octopaminergic neurons in addition to GABAergic neurons. Dopamine is synthesized from tyrosine by tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC), while octopamine is synthesized from tyrosine by AADC and tyramine β-hydroxylase (TBH). TH and TBH genes (*DjTH* and *DjTBH*, respectively) were previoiusly isolated from *Dugesia japonica* (Nishimura et al., 2007a; 2008b). Three types of DjTH-immunopositive neurons are differentially located in the dorsal brain, lateral branches,

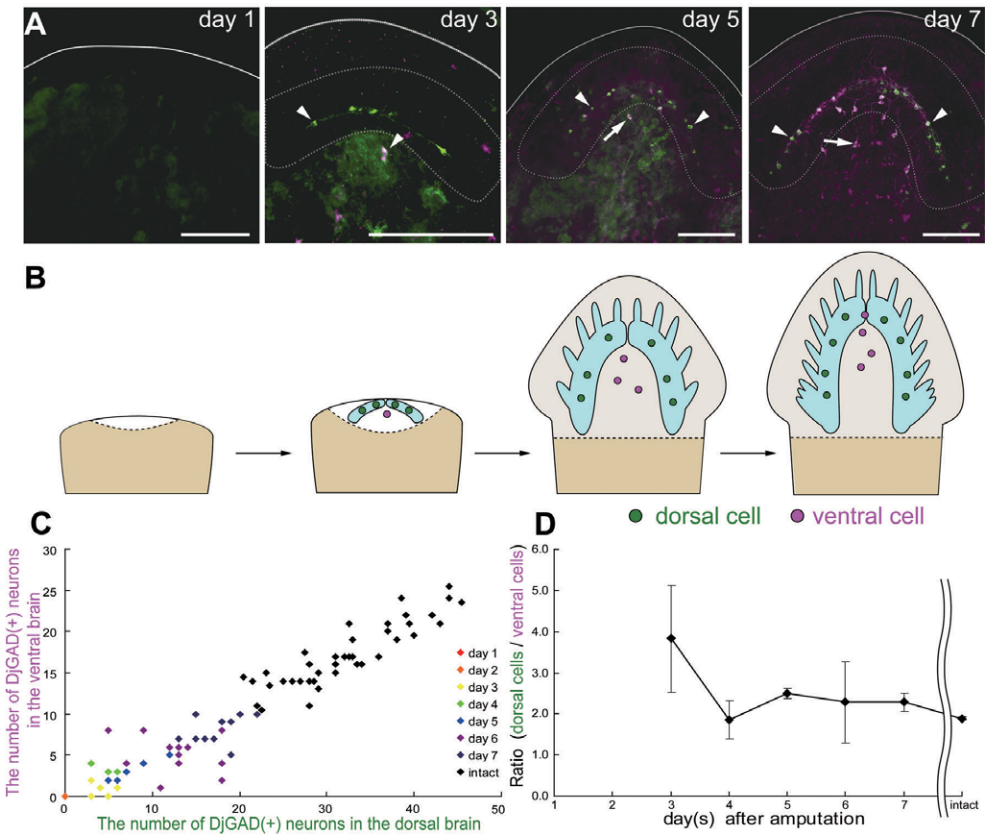

Fig. 7. Regeneration of DjGAD-immunopositive cells in the planarian brain. **(A)** DjGAD-immunopositive neurons stained 1, 3, 5, and 7 days after amputation. GABAergic neurons were detected after the third day. Scale bar: 150 μm. **(B)** Schematic diagram showing the regeneration of DjGAD (+) neurons in the planarian head. **(C)** Correlation between the numbers of dorsal and ventral DjGAD (+) neurons. Each point represents an individual planarian. **(D)** Ratio of dorsal to ventral GABAergic neurons during regeneration. Error bars, S.E.M.; n=5.

and VNCs, respectively (Fig. 6A). It has been shown that dopaminergic neurons are involved in the regulation of movement and behavior (Nishimura et al., 2007a). DjTBH-immunopositive neurons are distributed in the head, and commissure connections between several DjTBH (+) neurons were previously detected in the ventral part of the brain (Fig. 6A) (Nishimura et al., 2008b). We counted and plotted the numbers of all of these types of dopaminergic neurons and octopaminergic neurons in intact planarians (Fig. 6B). There was a statistically significant correlation between the total number of DjTH (+) neurons and DjTBH (+) neurons in the head. The ratio of the total number of DjTH (+) neurons to that of DjTBH (+) neurons was 2:1 in the intact animals. This suggests that the planarian brain maintains a constant ratio between different types of neurons.

Regeneration of the brain

We showed that the intact planarian brain can precisely maintain the ratios among various cell types regardless of differences in the location and type of cells. To reveal how this maintenance develops and is regulated, we investigated regeneration of the brain and eye.

With a high regenerative capability, planarians can completely regenerate the body within 7 days after amputation. In these studies, we cut the planarian body posterior to the pharynx and counted and plotted the number of GABAergic neurons in the brain during the course of regeneration from the tail fragment (Fig. 7A–C). During regeneration, DjGAD (+) neurons became detectable in the head about 3 days after amputation. The ratio of dorsal to ventro-medial immunopositive neurons at this stage was larger than 2:1, but gradually converged on 2:1 as the planarians regenerated (Fig. 7D). This means that the dorsal DjGAD (+) neurons begin to regenerate and increase first, but as both the dorsal and the ventro-medial DjGAD (+) neurons regenerate, the ratio of these two types finally becomes constant (2:1).

We also investigated the regeneration of octopaminergic and dopaminergic neurons (Fig. 8A–C). During regeneration, DjTBH (+) neurons were detected later than DjTH (+) neurons. From about 3 days after amputation, both these types of neurons began to appear in the head region, as with the DjGAD (+) neurons. At this time, the ratio of DjTH (+) neurons to DjTBH (+) neurons was larger than 2:1. As the planarians regenerated, however, this ratio gradually converged on 2:1, as with DjGAD (Fig. 8D). This means that DjTH (+) neurons increase faster than DjTBH (+) neurons early in regeneration, but that their numbers are regulated to achieve a final ratio of 2:1.

Regeneration of the cell ratio in the eye

It was shown above that as the planarian brain regenerates, the ratios between certain cell types change from greater than 2:1 to finally reach 2:1 with respect to location or cell-type (Figs. 7D, 8D). We also examined regeneration of the eye, which maintains a constant ratio between two different types of cells, similarly to the octopaminergic neurons and dopaminergic neurons in the brain. We showed that the eye in intact planarians is composed of twice as many visual neurons as pigment cells. To examine the time course of this ratio during regeneration, we counted and plotted the numbers of visual neurons and pigment cells during regeneration (Fig. 9A–C). Pigment cells began to appear on the second day after amputation, while both pigment cells and visual neurons had regenerated and formed a pair of small clusters on the third day. Early in regeneration, the ratio of visual cells to pigment cells was less than 2:1, in contrast to the maintenance of a constant ratio between different types of neurons in the brain during regeneration. At

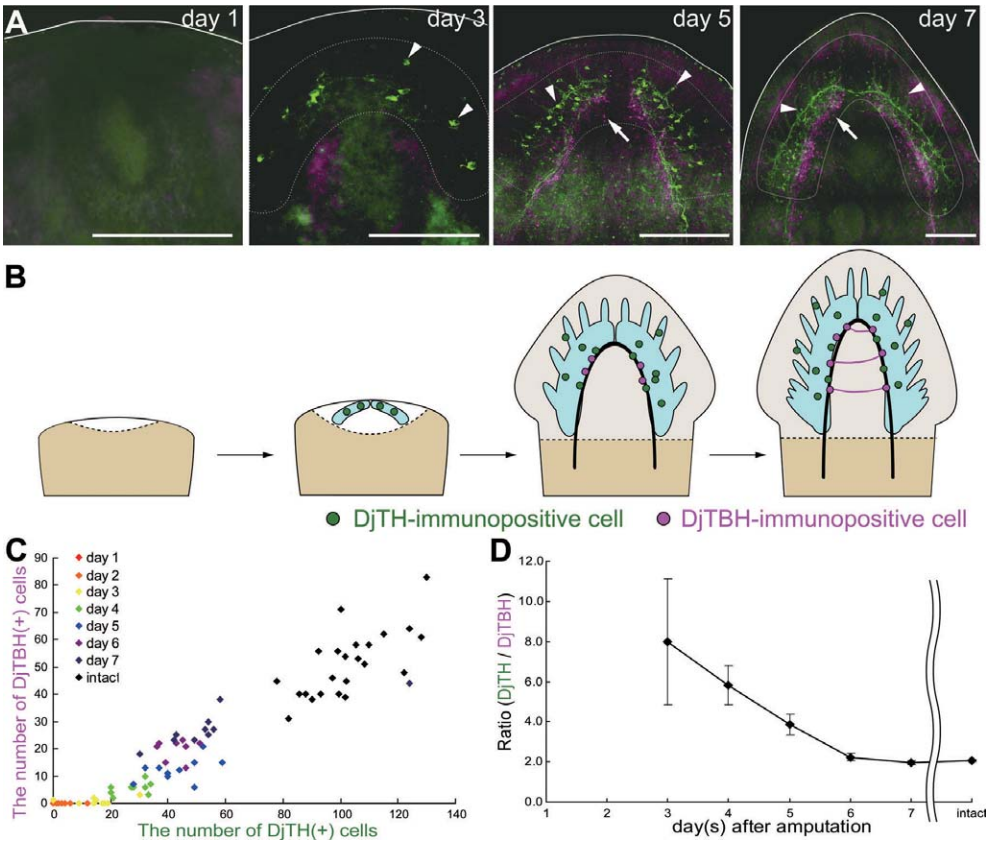

Fig. 8. Regeneration of DjTH- and DjTBH-immunopositive cells in the planarian brain. **(A)** Distribution of DjTH- and DjTBH-immunopositive neurons stained 1, 3, 5, and 7 days after amputation. Arrowheads indicate some dopaminergic neurons and arrows indicate some octopaminergic neurons. Scale bars: 150 μm. **(B)** Schematic diagram of the regeneration of DjTH (+) and DjTBH (+) neurons. **(C)** Correlation between the numbers of DjTH (+) and DjTBH (+) neurons. Each point represents an individual planarian. **(D)** Ratio of dopaminergic to octopaminergic neurons during regeneration. Error bars, S.E.M.; n>9.

5 or 6 days after amputation, the ratio of visual cells to pigment cells approached 2:1, which was the ratio in the intact animal (Fig. 9D).

Regulation of the number of cells generated from a common ancestral cell

The above results on the time course of the ratio between cell types during regeneration indicate that planarians may have a "counting mechanism" to regulate the numbers of cells of different types. To investigate this mechanism, we focused on an earlier stage of regeneration. After the third day following amputation, both visual cells and pigment cells could be detected at the protein level by immunostaining with the anti-Djarrestin or -DjTPH antibody, markers for visual neurons and pigment cells, respectively. Immunostaining for these markers revealed that some pigment cells but few visual cells existed early in regeneration (Day 2) (data not shown). To investigate an even earlier regeneration stage (before Day 2) at the mRNA level, we performed in-situ hybridization. In intact animals, visual neurons expressed *Djarrestin* mRNA and pigment cells expressed *DjTPH* mRNA (Fig. 10A). On the second day after amputation, cells co-expressing these two mRNAs were detected (Fig. 10B, C). These cells existed in pairs, and there was at most one pair per planarian. This result suggests that, in a single eye, the visual neurons and pigment cells differentiate from one common ancestral cell and that their number is regulated cell-autonomously, perhaps by a mechanism involving the timing of the cell cycle.

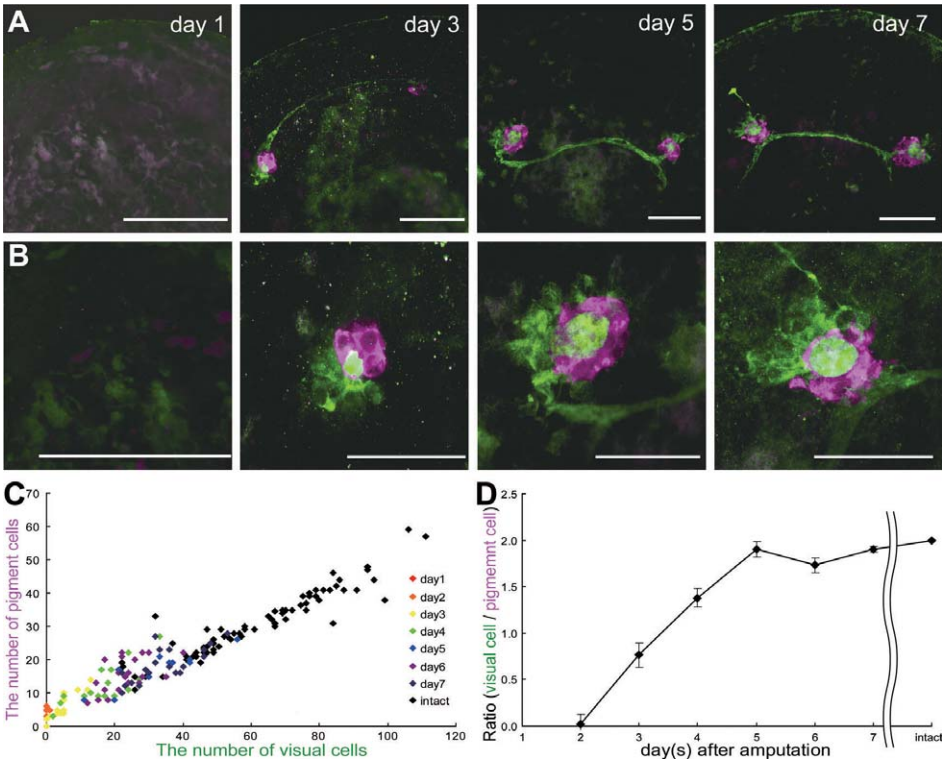

Fig. 9. Regeneration of the planarian eye. **(A)** Regeneration of the planarian eye visualized by immunostaining with anti-Djarrestin (green) and -DjTPH (magenta) 1, 3, 5 and 7 days after amputation. **(B)** More highly magnified views of the left eye in (A). **(C)** Correlation between the numbers of visual neurons and pigment cells. Each point represents an individual planarian. **(D)** Ratio of visual neurons to pigment cells during regeneration. Scale bars: 50 μm (A and B). Error bars, S.E.M.; n>5.

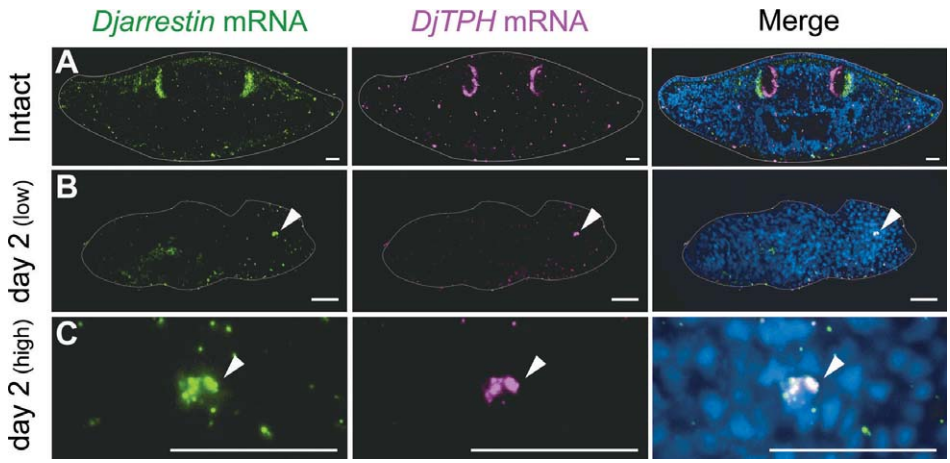

Fig. 10. Localizations of cells expressing *Djarrestin* and *DjTPH* mRNA in **(A)** an intact animal and **(B, C)** a regenerating animal 2 days after amputation. **(C)** More highly magnified views of (B). Blue indicates nuclei stained with Hoechst 33342. Scale bars: 30 μm.

DISCUSSION

Whole body proportions

Most animals maintain their body size and proportions during adulthood, although they change in body scale during development. However, here we showed that planarians can change their body length flexibly and proportionally by changing the number of cells. In intact *Dugesia japonica* (SSP), the proportion of the head size relative to the rest of the body is maintained constant (1:3) regardless of body length. Moreover, the change in size from small (2 mm) to large (9 mm) occurs quickly in planarians (in about 1 or 2 weeks) and proportionally. However, when planarians become too small (less than 1 mm) as a result of long-term starvation, they not only lose their normal body proportions, but also become inactive and show decreased negative phototaxis, sug-

gesting that the body proportion is important in maintaining individual functions and that about 10,000 neurons may be the minimum number of brain cells needed to maintain normal brain functions.

Number of specific cell types and body proportion

Classical quantitative analyses of cell types performed by maceration in other species of planarians, *Schmidtea mediterranea* and *Dugesia tigrina*, showed that planarians maintain a constant ratio of different cell types (Baguñá and Romero, 1981). The work performed here is an important advance because it used molecular markers to count specific cell types in intact animals, without maceration. The number of cells can be counted more precisely and specifically in non-dissociated samples than in maceration analyses. For example, we counted the visual and pigment cells in the eyes after staining with the anti-DjTPH and anti-Djarrestin antibodies, respectively, and found that the ratio of these two cell types is always about 2, even when the body size changes markedly. Using molecular markers, we also counted different types of neurons in the brain and discovered a regular distribution of these neurons in animals of different size. Thus, the numbers and distribution patterns of different neurons are homeostatically regulated by unknown mechanisms.

Tissue homeostasis

This study showed that planarians can change the number of cells, including neurons, in concert with body length. Studies using BrdU labeling (detecting proliferating cells) and TUNEL assays (detecting apoptotic cells) have revealed that some differentiated cells enter the apoptotic state and then are replenished by differentiation from neoblasts, even under physiological conditions (Hwang et al., 2004; Newmark and Sánchez, 2000). One might ask whether planarians can maintain coordinated movement after the number cells in the CNS changes. It is known that during tissue homeostasis, the planarian brain maintains fundamental functions such as controlling swimming and negative phototaxis. However, reward-learning behavior gradually fades starting about 2 weeks after conditioning and is completely lost after about 3 weeks (Inoue, unpublished). All of these results support the idea that the planarian brain renews neurons constantly by using the stem cell system and changing the number of cells.

Regulation of cell number

The results shown here clearly indicate that planarians have a “counting mechanism” to regulate the numbers of different cell types. How can planarians count the number of different types of cells? In the case of the eye, cells co-expressing *DjTPH* and *Djarrestin* mRNAs were detected during early regeneration, suggesting that two different types of cells differentiate from a common ancestor. Thus, some cell-autonomous mechanism such as the asymmetric division of stem cells may work to produce precise ratios of these different cell types. By contrast, we did not detect any common precursor or stem cells for neurons in the brain. During regeneration, the ratio among different neural cell types fluctuated in the early stage, but gradually returned to the original ratio in intact planarians. Some non-cell-autonomous mechanisms probably function to adjust the ratios among different types of neurons in the brain.

Molecules involved in regulating body proportions

There have been several reports on molecules involved in the regulation of body size. CHICO, a *Drosophila* homolog of vertebrate IRS1-4, plays an essential role in the control of cell size, cell number, and metabolism, suggesting that the insulin pathway controls the size of organs (Böhni et al., 1999; Hafen and Stocker, 2003). Also, recent studies in *Drosophila* revealed that genes related to the fat signaling pathway, such as *Fat*, *Expanded*, *Hippo*, and *Salvador*/*Shar-pei*, regulate proper tissue growth and development (Cho et al., 2006; Hamaratoglu et al., 2006; Shimizu et al., 2008; Silva et al., 2006; Pan, 2007). Moreover, it has been clarified that misexpression of a microRNA causes overgrowth in *Drosophila* (Nairz et al., 2006). We are now trying to identify homologs of these molecules from planarians and plan to conduct RNAi experiments involving these genes.

In addition to these molecules, we are also considering other genes for further RNAi studies. One is *nou-darake*, which encodes an FGFR-like molecule. It has been proposed that Nou-darake may function in the head in trapping an FGFR ligand molecule (which has tentatively been named factor X and might be actively secreted from the brain), and that a fraction of factor X might exit the head region (Agata and Umesono, 2008; Umesono and Agata, 2009). The ratio of trapped to leaked factor X may regulate the size of the head and the other parts of the body. This is a new hypothesis to explain how the proportions of the head and trunk parts are maintained constant. As the size of the brain increases, the amount of factor X leaving the head might increase and stimulate growth of organs in the trunk. Thus, the brain may have the capacity to determine the size of other organs in planarians. *DjWntA* is another candidate that might determine the size of the brain. *DjWntA*-RNAi planarians form a posteriorly expanded brain (Kobayashi et al., 2007). These signaling molecules, which are predicted to have activities as growth factors, may have a crucial role in regulating body and organ proportions. In the future, we should carefully count cell numbers in planarians with RNAi knockdowns of these genes. We are also planning a non-biased screening of genes involved in the regulation of body proportions by a combination of RNAi and immunostaining like that used in this analysis.

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REFERENCES

Agata K, Umesono Y (2008) Brain regeneration from pluripotent stem cells in planarian. Phil Trans R Soc B 363: 2071–2078

Agata K, Soejima Y, Kato K, Kobayashi C, Umesono Y, Watanabe K (1998) Structure of the planarian central nervous system (CNS) revealed by neuronal cell markers. Zool Sci 15: 433–440

Asano Y, Nakamura S, Ishida S, Azuma K, Shinozawa T (1998) Rhodopsin-like protein in planarian eye and auricle: detection

and functional analysis. J Exp Biol 201: 1263–1271
Baguñá J, Romero R (1981) Quantitative analysis of cell types during growth, degrowth and regeneration in the planarians *Dugesia mediterranea* and *Dugesia tigrina*. Hydrobiologia 84: 181–194
Böhni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andruss BF, Beckingham K, Hafen E (1999) Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4. Cell 97: 865–875
Cebri F (2008) Organization of the nervous system in the model planarian *Schmidtea mediterranea*: an immunocytochemical study. Neurosci Res 61: 375–384
Cebrià F, Kobayashi C, Umesono Y, Nakazawa M, Mineta K, et al. (2002) FGFR-related gene *nou-darake* restricts brain tissues to the head region of planarians. Nature 419: 620–624
Cho E, Feng Y, Rauskolb C, Maitra S, Fehon R, Irvine KD (2006) Delineation of a Fat tumor suppressor pathway. Nat Genet 38: 1142–1150
Dowar BL (1973) A combined relaxing agent and fixative for triclads (planarians). Stain Technol 48: 93–94
Hafen E, Stocker H (2003) How are the sizes of cells, organs, and bodies controlled? PLoS Biol 1: 319–323
Hamaratoglu F, Willecke M, Kango-Singh M, Nolo R, Hyun E, Tao C, Jafar-Nejad H, Halder G (2006) The tumour-suppressor genes *NF2*/*Merlin* and *Expanded* act through *Hippo* signalling to regulate cell proliferation and apoptosis. Nat Cell Biol 8: 27–36
Hwang JS, Kobayashi C, Agata K, Ikeo K, Gojobori T (2004) Detection of apoptosis during planarian regeneration by the expression of apoptosis-related genes and TUNEL assay. Gene 333: 15–25
Inoue T, Kumamoto H, Okamoto K, Umesono Y, Sakai M, Sánchez AA, Agata K (2004) Morphological and functional recovery of the planarian photosensing system during head regeneration. Zool Sci 21: 275–283
Ito H, Saito Y, Watanabe K, Orii H (2001) Epimorphic regeneration of the distal part of the planarian pharynx. Dev Genes Evol 211: 2–9
Kobayashi C, Kobayashi S, Orii H, Watanabe K, Agata K (1998) Identification of two distinct muscles in the planarian *Dugesia japonica* by their expression of myosin heavy chain genes. Zool Sci 15: 861–869
Kobayashi C, Saito Y, Ogawa K, Agata K (2007) Wnt signaling is required for antero-posterior patterning of the planarian brain. Dev Biol 15: 714–724
Nairz K, Rottig C, Rintelen F, Zdobnov E, Moser M, Hafen E (2006) Overgrowth caused by misexpression of a microRNA with dispensable wild-type function. Dev Biol 291: 314–324
Nakazawa M, Cebrià F, Mineta K, Ike K, Agata K, Gojobori T (2003) Search for the evolutionary origin of a brain: planarian brain characterized by microarray. Mol Biol Evol 20: 784–791
Newmark PA, Sánchez AA (2000) Bromodeoxyuridine specifically labels the regenerative stem cells of planarians. Dev Biol 220: 142–153
Nishimura K, Kitamura Y, Inoue T, Umesono Y, Sano S, et al. (2007a) Reconstruction of dopaminergic neural network and locomotion function in planarian regenerates. Dev Neurobiol 67: 1059–1078
Nishimura K, Kitamura Y, Inoue T, Umesono Y, Yoshimoto K, Taniguchi T, Agata K (2007b) Identification and distribution of *tryptophan hydroxylase* (TPH)-positive neurons in the planarian *Dugesia japonica*. Neurosci Res 59: 101–106
Nishimura K, Kitamura Y, Umesono Y, Takeuchi K, Takata K, Taniguchi T, Agata K (2008a) Identification of glutamic acid decarboxylase gene and distribution of GABAergic nervous system in the planarian *Dugesia japonica*. Neuroscience 153: 1103–1114
Nishimura K, Kitamura Y, Inoue T, Umesono Y, Yoshimoto K, Taniguchi T, Agata K (2008b) Characterization of tyramine beta-hydroxylase in planarian *Dugesia japonica*: cloning and expression. Neurochem Int 53: 184–192
Okamoto K, Takeuchi K, Agata K (2005) Neural projections in planarian brain revealed by fluorescent dye tracing. Zool Sci 22: 535–546
Oviedo NJ, Newmark PA, Sánchez AA (2003) Allometric scaling and proportion regulation in the freshwater planarian *Schmidtea mediterranea*. Dev Dyn 226: 326–333
Pan D (2007) Hippo signaling in organ size control. Genes Dev 21: 886–897
Romero R, Baguñá J (1991) Quantitative cellular analysis of growth and reproduction in freshwater planarians (Turbellaria; Tricladida). I. A cellular description of the intact organism. Invertebr Reprod Dev 19:157–165
Sakai F, Agata K, Orii H, Watanabe K (2000) Organization and regeneration ability of spontaneous supernumerary eyes in planarians — eye regeneration field and pathway selection by optic nerves. Zool Sci 17: 375–381
Shimizu T, Ho LL, Lai ZC (2008) The *mob as tumor suppressor* gene is essential for early development and regulates tissue growth in *Drosophila*. Genetics 178: 957–965
Silva E, Tsatskis Y, Gardano L, Tapon N, McNeill H (2006) The tumor-suppressor gene *fat* controls tissue growth upstream of expanded in the hippo signaling pathway. Curr Biol 16: 2081–2089
Takano T, Pulvers JN, Inoue T, Tarui H, Sakamoto H, Agata K, Umesono Y (2007) Regeneration-dependent conditional gene knockdown (Readyknock) in planarian: demonstration of requirement for *Djsnap-25* expression in the brain for negative phototactic behavior. Dev Growth Differ 49: 383–394
Tazaki A, Gaudieri S, Ikeo K, Gojobori T, Watanabe K, Agata K (1999) Neural network in planarian revealed by an antibody against planarian synaptotagmin homologue. Biochem Biophys Res Comm 260: 426–432
Umesono Y, Agata K (2009) Evolution and regeneration of the planarian central nervous system. Dev Growth Differ 51: 185–195
Umesono Y, Watanabe K, Agata K (1997) A planarian *orthopedia* homolog is specifically expressed in the branch region of both the mature and regenerating brain. Dev Growth Differ 39: 723–727
Umesono Y, Watanabe K, Agata K (1999) Distinct structural domains in the planarian brain defined by the expression of evolutionarily conserved homeobox genes. Dev Genes Evol 209: 31–39

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