Brain regeneration from pluripotent stem cells in planarian

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How can planarians regenerate their brain? Recently we have identified many genes critical for this process. Brain regeneration can be divided into five steps: (1) anterior blastema formation, (2) brain rudiment formation, (3) pattern formation, (4) neural network formation, and (5) functional recovery. Here we will describe the structure and process of regeneration of the planarian brain in the first part, and then introduce genes involved in brain regeneration in the second part. Especially, we will speculate about molecular events during the early steps of brain regeneration in this review. The finding providing the greatest insight thus far is the discovery of the *nou-darake* (*ndk*; 'brains everywhere' in Japanese) gene, since brain neurons are formed throughout the entire body as a result of loss of function of the *ndk* gene. This finding provides a clue for elucidating the molecular and cellular mechanisms underlying brain regeneration. Here we describe the molecular action of the *nou-darake* gene and propose a new model to explain brain regeneration and restriction in the head region of the planarians.

Keywords: brain; regeneration; planarian; pluripotent stem cell; *nou-darake*

1. INTRODUCTION

Planarians can regenerate an entire body including a brain from a small piece of the body in which no brain tissues remain. How can planarians regenerate the brain from a small fragment of almost any portion of the body? It is believed that this is accomplished by pluripotent stem cells that are distributed throughout the body, which can give rise to all types of cells, including brain neurons (Agata & Watanabe 1999; Newmark & Sánchez Alvarado 2002; Saló & Baguña 2002; Reddien & Sánchez Alvarado 2004; Agata *et al.* 2006; Saló 2006). However, we do not know the process by which the brain is regenerated or the genes involved in brain regeneration. Recently, PCR-based cloning, expressed sequence tag (EST) projects and DNA chip analyses have succeeded in identifying many genes expressed during the process of brain regeneration (Agata *et al.* 1998; Tazaki *et al.* 1999; Cebrià *et al.* 2002a–c; Sánchez Alvarado *et al.* 2002; Nakazawa *et al.* 2003; Mineta *et al.* 2003; Agata 2003). In addition, a gene-knockdown method using RNA interference (RNAi) in planarians has been developed (Sánchez Alvarado & Newmark 1999; Newmark *et al.* 2003; Orii *et al.* 2003). Recently, we have succeeded in conditional gene knockdown by the combination of RNAi and the regeneration ability of planarians, named 'Readyknock' (Takano *et al.* 2007). The availability of these methods has greatly facilitated our ability to investigate the genetic programmes underlying brain regeneration. Here, recent progress in the study of planarian brain regeneration will be reviewed.

2. PLURIPOTENT STEM CELLS IN PLANARIANS

The planarian is a very unique animal that maintains pluripotent stem cells, referred to as 'neoblasts', in adulthood (Baguña 1981). The neoblasts are distributed throughout the entire region of the body. This pluripotent stem cell system is essential for both asexual and sexual reproduction, and also supports the high regenerative ability of planarians (Agata *et al.* 2006). Although the pluripotency of planarian stem cells has not yet been demonstrated by clonal cell culture or single-cell transplantation experiments, these cells have long been believed to exist owing to the extreme regenerative ability of planarians. The neoblasts can be identified not only by their gross morphology but also by unique cytoplasmic components, called chromatoid bodies (Morita *et al.* 1969). The chromatoid body is an electron-dense particle similar to germ plasm, which is a cytoplasmic component specific to germ cells (Shibata *et al.* 1999). However, its function is not yet understood. The neoblasts are specifically eliminated by X-ray irradiation, and X-ray irradiated planarians lose their regenerative ability (Wolff & Dubois 1948). Partial X-ray irradiation experiments demonstrated that stem cells located in any part of the body can rescue different irradiated body parts, suggesting that stem cells may have equivalent pluripotentiality. Recently, we have purified X-ray-sensitive cells using fluorescence-activated cell sorter (FACS) sorting and characterized them at the molecular and cellular levels (Hayashi *et al.* 2006). These molecular and morphological analyses suggested both pluripotency and heterogeneity of the planarian stem cells (Higuchi *et al.* 2007).

3. STRUCTURE OF THE PLANARIAN BRAIN

Most people might suppose that planarians are able to regenerate their brain because its structure is quite

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Figure 1. Structure of the planarian CNS. (a) A whole mount *in situ* hybridization view from the ventral side showing the gross structure of the planarian CNS (stained with a prohormone convertase 2 homologue, *DjPC2*, probe). The cell bodies of neurons are stained. The planarian CNS is composed of an inverted U-shaped brain (bracket) and a pair of VNCs (arrows). (b) Immunostaining of the planarian nervous system with anti-DjSYT (planarian synaptotagmin). In contrast to the pattern in (a), only axons are stained. The ladder-like structure of the commissure neurons of the VNCs can be clearly observed (ventral view).

simple. However, our recent studies have revealed that planarians have a more complex brain than we had expected. The planarian central nervous system (CNS) is composed of a two-lobed brain and a pair of ventral nerve cords (VNCs; figure 1). In the old literature, these two structures were drawn as a united structure, but our anatomical studies using molecular markers clearly indicated that the brain is actually an independent structure from the VNCs. The brain is located on the dorsal side, in contrast to the VNCs, which are connected to each other on the ventral side of the brain (Agata *et al.* 1998; Cebrià *et al.* 2002c). The planarian brain has an inverted U-shaped structure with nine branches on each outer side (figure 2). Two eyes are located on the dorsal side at the level of the third branch, and visual axons form an optic chiasma on the dorsal-internal region of the brain (Sakai *et al.* 2000). The sixth to ninth branches cluster more closely and form auricles on the surface, which may function as the sensory organ of taste. We also found that these structurally distinct diverse domains are defined by the discrete expression of three evolutionarily conserved homeobox genes, *DjotxA*, *DjotxB* and *Djotp* (Umesono *et al.* 1997, 1999). Interestingly, expression analysis of these genes via EST projects and DNA microarray hybridization strongly suggests that the expression domains defined by the three homeobox genes may actually correspond to functional domains (figure 2). Photosensory and chemosensory neurons are formed in the *DjotxA*- and *Djotp*-expressing domains, respectively. Mechanosensory neurons are found in a cephalic region that is devoid of the expression of the three *otd/Otx*-related genes (Mineta *et al.* 2003; Nakazawa *et al.* 2003). We investigated precisely how the neurons in each domain are connected by analysing the projection patterns of each domain in detail by DiI/DiD tracing (Okamoto *et al.* 2005). Also, we found that a planarian *netrin*

Figure 2. Domain structure of the planarian brain. The planarian brain can be divided into at least four structurally and functionally different domains, which are defined by the discrete expression of three *otd/Otx* family genes. The planarian brain is composed of two main lobes with lateral branches (nine asterisks). Lateral branch neurons are defined by *Djotp* expression (green) and are composed of chemosensory neurons. Both photosensory neurons and their target region in the brain are defined by *DjotxA* expression (blue). The two main lobes, where a variety of interneurons have been identified, are defined by *DjotxB* expression (red). Mechanosensory neurons (yellow) are formed in the peripheral region of the head, which is defined by being devoid of the *Djotx* expression of the three *otd/Otx*-related genes. In summary, a variety of external signals are sensed by the sensory neuron clusters in the head region, and the signals are integrated in the *DjotxB*-positive main lobes and transmitted to various regions of the body after processing via the pair of VNCs that are connected to the ventral side of the brain. pc, Pigmented eyecups.

homologue (*Djnetrin*) is expressed in the junctions between visual neurons and the brain as well as between the VNCs and the brain (Cebrià *et al.* 2002c; Cebrià & Newmark 2005). These analyses suggested that a variety of external signals received by these sensory neurons may be integrated in the *DjotxB*-expressing domain, where dopaminergic neurons are concentrated (Nishimura *et al.* 2007), and that these integrated signals may be transferred to the body muscles through the VNCs (Tazaki *et al.* 1999). Also, additional functional domains in the planarian brain have been defined by gene expression analyses, and give us a more complex view of this organ than suggested by its relatively simple morphology (Cebrià *et al.* 2002a).

4. THE REGENERATION PROCESS

How can planarians regenerate such a sophisticated brain within 5 days of amputation? The process of regeneration of the brain has been extensively analysed by whole-mount staining. The brain is formed in the anterior blastema. Interestingly, the brain regenerates independently from the VNCs remaining in the stump, and becomes connected to them approximately 3–4 days after regeneration. The process of brain regeneration can be divided into five

Figure 3. Summary of brain regeneration process. Planarian brain regeneration can be divided into five steps, as indicated by the histological observations and gene expression patterns: (1) anterior blastema formation, (2) brain rudiment formation, (3) pattern formation, (4) neural network formation, and (5) functional recovery. The following genes and signalling systems are involved in these steps: step 1, BMP/*noggin* signal; step 2, *nou-darake*/FGFR signal; step 3, Wnt signal and *otd/Otx* family genes; step 4, *netrins* and CAMs; and step 5, 1020HH and eye 53, which were identified as genes involved in this step by RNAi (Inoue *et al.* 2004).

steps (Cebrià *et al.* 2002c): (1) anterior blastema formation, (2) brain rudiment formation, (3) pattern formation, (4) neural network formation, and (5) functional recovery (figure 3). The anterior blastema is formed after wound closure (step 1; Shibata *et al.* 1999; Hwang *et al.* 2004) and then a brain rudiment is formed in the anterior blastema (step 2; Cebrià *et al.* 2002b). These first two steps occur within 24 hours after amputation and it is very difficult to distinguish between them (see below). The earliest gene known to be activated after amputation is a *noggin*-like gene, *Djnlga*, the transcript of which can be detected by *in situ* hybridization 8 hours after amputation. This gene is activated by dorsal–ventral interaction after wound closure (Kato *et al.* 1999, 2001; Ogawa *et al.* 2002b). Interaction of BMP and *noggin* may have an important role in inducing blastema formation (Orii *et al.* 1998; Agata *et al.* 2003). After formation of the blastema, body regionality along the anterior–posterior (A–P) axis is reorganized by A–P intercalation (Kobayashi *et al.* 1999a,b; Orii *et al.* 1999; Agata *et al.* 2003, 2007), and the brain rudiment is formed in the anterior blastema. After formation of the brain rudiment, the regenerating brain starts to undergo pattern formation (step 3), in which the expression of three different *otd/Otx*-related genes is detected 36 hours after amputation (Umesono *et al.* 1997, 1999). While we have not been able to distinguish the expression domains of these three *otd/Otx*-related genes at the initial stage of pattern formation, their expression domains gradually become segregated along the A–P axis. Then the midline region of the brain rudiment protrudes, forming an inverted U-shaped brain. A transcription factor, *DjFoxG* (*BF2* homologue), is expressed in the tip of the inverted U-shaped brain (Koinuma *et al.* 2000, 2003; Saito *et al.* 2003). Expression of the *Djotp* gene is restricted to the lateral region of the brain, which consists of branched structures composed of chemosensory neurons, and becomes evident approximately 3 days after regeneration. A *Wnt* homologue gene is also activated in the posterior region of the brain rudiment

at the late stage of pattern formation (Kobayashi *et al.* 2007). In step 4, *netrin* homologues begin to be expressed, and then the eyes and brain, and the brain and VNCs become connected to each other (Cebrià *et al.* 2002a; Cebrià & Newmark 2005). *N-CAM* and *cadherin* family genes are also activated during this period (Fusaoka *et al.* 2006). The network structure of the CNS is completely reformed within 4 days. Although morphological recovery is completed within 4 days, an additional day is needed for functional recovery. Planarians show negative phototactic behaviour. By using a phototaxis assay system that we developed to analyse light response recovery during head regeneration, we found that light evasion is robustly re-established 5 days after amputation. Interestingly, several genes are activated at the late stage of brain regeneration, suggesting that these genes may be involved in functional recovery (Cebrià *et al.* 2002a; Inoue *et al.* 2004). In conclusion, brain regeneration proceeds in a sequential manner accompanied by appropriate changes of gene expression, like embryogenesis.

5. SCREENING OF GENES INVOLVED IN THE BRAIN REGENERATION

To identify genes involved in brain formation and function, we constructed a cDNA library of the head region of planarians and sequenced 10 000 randomly picked clones in a collaborative study with Dr Gojobori's group at the National Institute of Genetics, Mishima, Japan. In this project, 3351 non-redundant genes were identified and 145 genes were classified as nervous system-related genes by sequence analyses and homology searches (Mineta *et al.* 2003). To further identify brain-specific or -abundant genes, differential screening between head and trunk region-specific probes was performed using a DNA chip that we constructed, on which non-redundant genes were spotted. From these analyses, 216 genes were identified as head-abundant genes (Nakazawa *et al.* 2003). We then checked the expression patterns of these genes in intact and regenerating brains by whole-mount *in situ*

hybridization. Interestingly, most of these genes are expressed in distinct domains of the intact brain and at distinct stages during the brain regeneration. They can be classified into four classes according to their expression timing during the process of brain regeneration: early; early-middle; late-middle; and late genes (Cebrià *et al.* 2002c). Early expressed genes may be involved in the first and second steps of brain regeneration. Early- and late-middle-expressed genes may have functions important for pattern and network formations, respectively. The most curious genes are the late-expressed genes. Several genes start to be expressed after the completion of the structural regeneration. To investigate the function of these genes at each step of regeneration, we conducted RNAi analyses using the methodology developed by Alejandro Sánchez Alvarado's group. Although we have identified several genes affecting brain functions and planarian behaviours (Inoue *et al.* 2004; Fusaoka *et al.* 2006; Nishimura *et al.* 2007; Takano *et al.* 2007), we could not identify genes interfering with brain regeneration for a long time. This is probably due, in part, to the fact that redundant genes may often act in the process of brain regeneration to maximize the likelihood of its success. However, we recently succeeded in identifying a gene essential for brain regeneration, namely, a clathrin heavy chain gene (Inoue *et al.* 2007). When we knocked down the *DjCHC* gene (planarian homologue of a clathrin heavy chain gene) by RNAi, brain regeneration could not occur. We then carefully investigated the functional target point of the *DjCHC* gene. Interestingly, the early steps of brain regeneration, including brain rudiment formation and patterning, were not disturbed in the *DjCHC*-RNAi planarians. However, the projection of axons during CNS regeneration was strongly disturbed after proper brain patterning. Finally, using primary cultures of planarian neurons purified by FACS after RNAi treatment, we succeeded in clearly showing that the *DjCHC* gene was not essential for neural differentiation, but was required for neurite extension and maintenance, and that *DjCHC*-RNAi-treated neurons entered an apoptotic state (Inoue *et al.* 2007). These results suggested that clathrin-mediated endocytic signals may be required not only for the maintenance of neurons after synaptic formation, but also for axonal extension at the early stage of brain regeneration. Recently, we also succeeded in demonstrating by RNAi that Wnt signalling is involved in brain patterning along the A–P axis in planarians, as in vertebrates (Kobayashi *et al.* 2007).

6. DISCOVERY OF *nou-darake* (*ndk*) GENE

The most striking result of the RNAi studies was obtained when we made knockdown planarians of a gene designated gene 721HH. This gene had been identified as the one showing the highest head/trunk ratio in DNA chip analysis and showed head-specific expression in whole-mount RNA hybridization (Nakazawa *et al.* 2003). Gene 721HH was activated at a very early stage of the brain rudiment formation. Therefore, we expected that knockdown planarians of this gene might not be able to regenerate the brain; however, a completely opposite result was obtained.

Figure 4. Brains are ectopically formed in all regions of the body in *nou-darake* RNAi planarians. Pluripotent stem cells distributed throughout the body give rise to brains in *nou-darake* RNAi planarians. The *nou-darake* gene encodes an FGFR-like molecule lacking a tyrosine kinase domain in its intracellular region and functions to restrict brain formation in the head region. (a) A control animal stained with a brain-specific glutamate receptor gene probe (1008HH). (b–d) The *nou-darake* RNAi planarians stained with the same probe forming ectopic eyes and brains in the posterior region of the bodies.

Figure 5. Inhibitor model. (a) The regenerating brain may secrete an inhibitor molecule that suppresses the differentiation of brain neurons in the trunk region. The brain inhibitor is indicated in red. (b) Loss of the brain inhibitor would allow the ectopic differentiation of brain neurons in the trunk region.

Gene 721HH-knockdown planarians regenerated the brain without any disturbance of the brain formation. They formed the brain and eyes normally. However, one week after regeneration, they began to form ectopic eyes in the trunk region. Interestingly, ectopic brains were also formed in these animals in addition to the eyes (figure 4). We then investigated whether these ectopic brains had the same domain structures as normal brains. Amazingly, all of the ectopic brains contained *DjotxA*-, *DjotxB*- and *Djotp*-positive domains, and *Djnetrin* was expressed in the junctions between the ectopic brains and the ectopically formed visual neurons as well as between the ectopic brain and VNCs, suggesting that the complete brain structures

Figure 6. Interpretation of *nou-darake* function and its RNAi phenotype. (a) Capture model proposed in this review: a brain activator (which has not yet been identified) may stimulate the differentiation of brain neurons, but may be captured by NOU-DARAKE (NDK) and consequently not able to diffuse outside the head region. The following three experiments support the capture model: *Xbra* expression and gastrulation were inhibited in the planarian *nou-darake* mRNA-injected embryos; induction of *Xbra* expression in the animal cap by bFGF administration was completely suppressed in planarian *nou-darake* mRNA-injected animal caps; ectopic brain formation of *nou-darake* RNAi planarians was suppressed by co-injection of double-stranded RNAs of two FGFR homologue genes, *DjFGFR1/2*, which were specifically expressed in X-ray-sensitive stem cells. Brain activator and NDK are indicated in green and red, respectively. (b) In intact planarians, excess brain activators are trapped by NDK, but they can diffuse to the posterior region of the body in *nou-darake* RNAi planarians. (c) In triple-knockdown planarians co-injected with the two *FGFR1/2* double-stranded RNAs in addition to *nou-darake* RNAs, ectopic brains were not formed in the trunk region due to the lack of expression of FGFR in the stem cells. The weak points of this model are that we need to postulate the existence of a third FGFR molecule (*DjFGFR3*), and that we have not yet identified any brain activator molecules (see text).

were formed in the ectopically formed brains (Cebrià *et al.* 2002b). Based on these observations, we named gene 721HH as *nou-darake*, meaning 'brains everywhere' in Japanese.

7. MOLECULAR ACTION OF *ndk*

Classical experiments suggested that the planarian head region is a source of diffusible factor that inhibits extra brain formation during regeneration (inhibitor model; figure 5; Lender 1956). Initially, we believed that we had succeeded in identifying such a diffusible inhibitor. However, structural analysis of the putative protein encoded by the *ndk* gene revealed discrepancies with our initial expectation. *ndk* Encodes a trans-membrane protein possessing two immunoglobulin (Ig)-like domains in its extracellular region, which show high similarity to the corresponding domains of human FGFR3, but lacking the cytoplasmic kinase domains characteristic of this receptor family (Cebrià *et al.* 2002b). Recently we have succeeded in producing an antibody against the extracellular domain of NDK and detected strong signals of NDK in brain cells (Ishizawa *et al.* 2003, unpublished observation). These observations suggest that NDK functions on the cell surface of the brain cells without diffusion.

How does NDK inhibit brain formation in the trunk region, although it cannot diffuse to the posterior region? Important results in this regard were obtained by combinatory gene-knockdown experiments and mRNA injection into *Xenopus* embryos. Before discovering *ndk*, we had already identified two FGFR-like molecules from planarian, named *DjFGFR1* and

DjFGFR2, and characterized them in detail (Ogawa *et al.* 1998, 2002a). Both of them are expressed in X-ray-sensitive, stem cells. While gene-knockdown planarians of either of them did not cause any clear defects in regeneration, triple-knockdown planarians of these two genes plus *ndk* suppressed the *nou-darake* phenotype (Cebrià *et al.* 2002b), indicating that ectopic brains are formed through FGFR signalling. Interestingly, when planarian *ndk* mRNA was injected into *Xenopus* embryos, it inhibited gastrulation by interfering with *Xbra* expression (Cebrià *et al.* 2002b), one of the target genes of FGF signalling. The effects of *ndk* were still observed when both its intracellular and transmembrane regions were deleted, indicating that the extracellular domain of NDK is sufficient for the inhibition of the *Xbra* expression. This neutralizing activity was also confirmed by animal cap assays. Exogenously administered FGF was neutralized by NDK (Cebrià *et al.* 2002b). These results suggest that NDK may have the ability to bind to FGF or FGF-like molecule(s) and modulate FGF signalling. Such an FGF-like molecule might function as a brain activator in planarians.

8. ALTERNATIVE 'CAPTURE' MODEL TO EXPLAIN NDK ACTION

Based on these observations we propose the following speculative model, named the 'capture model' (figure 6). NDK is specifically expressed in the head region and may regulate the diffusion range of brain activators (FGF or FGF-like molecules) from a putative source in the head region to the rest of the

body through direct interaction with brain activators. Loss of function of *ndk* would allow these factors to travel to more posterior regions, and thus activate FGF receptors outside the head region to trigger ectopic brain formation (figure 6). Our observation of gradual brain expansion to more posterior regions in dsRNA-injected animals supports this idea. In this situation, these hypothetical brain activators must travel over considerable distances of several millimetres between the planarian head and the posterior regions where the ectopic brain is formed. Some facilitated transport of brain activators will be essential for this model. The VNCs are one of the candidates for mediators of the facilitated transport of brain activators. The weak points of this model are that double *DjFGFR1/DjFGFR2* dsRNA-injected animals could regenerate the normal brain structure (Ogawa *et al.* 2002a), indicating that the activity of DjFGFR1 and DjFGFR2 is not sufficient for the original brain formation. Thus, we need to postulate the existence of a third FGFR molecule (DjFGFR3) that may compensate for the DjFGFR1 and DjFGFR2 functions in the double *DjFGFR1/DjFGFR2* dsRNA-injected animals (figure 6c). However, we have not yet identified any brain activator molecules or DjFGFR3.

9. FGF SIGNALS AND BRAIN FORMATION

ndk provides strong molecular evidence for the existence of a brain-inducing circuit based on an FGF signalling pathway in planarians. While antagonists of BMP4 are believed to be the major neural inducers in vertebrates, recent work has implicated FGFs as key conserved mediators of neural induction (Launay *et al.* 1996; Streit *et al.* 2000; Wilson *et al.* 2000; Wilson & Edlund 2001; Akai & Storey 2003), suggesting that FGF ligands may be essential for neural induction during evolution. However, we are yet to identify FGF-like ligands in planarians. Therefore, identification of the FGF-like ligands is one of the crucial future research goals in planarians.

The fact that planarian *ndk* can functionally inhibit *Xbra* activation during *Xenopus* gastrulation raises the possibility that the vertebrate homologue of *ndk* may play a role in modulating FGF signalling. Further studies are required to clarify the extent of *ndk*'s involvement in vertebrate organogenesis, in particular neurogenesis. A transmembrane receptor, FGFR1, in vertebrates exhibits striking similarity of its domain structures to that of NDK. FGFR1 contains three extracellular Ig-like domains related to FGF receptors and lacks the cytoplasmic kinase domain (Wiedemann & Trueb 2000). Recently, some of our collaborators, Dr Taira's group, identified *FGFRL1* from *Xenopus* (*XFGFRL1*) and analysed its expression pattern during embryogenesis (Hayashi *et al.* 2004). Interestingly, *XFGFRL1* is first expressed specifically in the anterior region of embryos and is coexpressed with *XFGF8* in many regions throughout embryogenesis. These findings strongly suggest that *ndk/FGFRL1* may play a role in the anterior specification in various animals by modulating FGF signalling. Further studies will be required to understand the molecular action of *XFGFRL1* during embryogenesis.

10. EVOLUTIONARY IMPLICATIONS

During the process of investigating brain regeneration in planarians, we realized that planarians have a more highly organized brain than we had expected, as shown above. We found that the gross structure of the planarian CNS along the axis is strikingly similar to the distribution pattern of the 'primary' neurons of vertebrate embryos, which differentiate at the neural plate stage to produce a fundamental nervous system, although the vertebrate CNS is located on the opposite side of the planarian CNS (Agata *et al.* 1998). These data suggest that the basic plan for CNS development along the A–P axis might have been acquired at an early stage of evolution before conversion of the location of the CNS from the ventral to the dorsal side. Based on these observations, we recently proposed that drastic evolution of the brain may have accompanied the evolutionary emergence of the neural stem cell system, which enables the development of a variety of neurons from single cells (Agata *et al.* 2006).

11. CONCLUSIONS AND PROSPECTS

Planarians have provided many novel insights into brain regeneration and evolution. Especially, the gene-knockdown method using RNAi has greatly facilitated our ability to investigate the genetic programmes underlying brain development and function by testing and observing the effects of specifically targeted genes during brain regeneration (Sánchez Alvarado 2006).

The most important advantage of using planarians is that the planarian brain can regenerate from pluripotent stem cells distributed throughout the body (Agata & Watanabe 1999; Agata *et al.* 2006). Although a brain cannot be generated at present from mouse embryonic stem (ES) cells, planarians can form a brain from pluripotent stem cells from any portion of the body within one week of amputation. Studying brain regeneration in planarians may provide unique information enabling the generation of a brain from mouse or other vertebrate ES cells *in vitro* in the near future. For this purpose, we are attempting to purify both brain neurons and pluripotent stem cells from planarians using FACS (Asami *et al.* 2002; Hayashi *et al.* 2006), and to clarify whether pluripotent stem cells differentiate into neurons directly, or indirectly via a neural stem cell state (Agata *et al.* 2006).

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