A LIM-homeobox gene is required for differentiation of Wnt-expressing cells at the posterior end of the planarian body

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SUMMARY
Planarians have high regenerative ability, which is dependent on pluripotent adult somatic stem cells called neoblasts. Recently, canonical Wnt/β-catenin signaling was shown to be required for posterior specification, and Hedgehog signaling was shown to control anterior-posterior polarity via activation of the Djwnt1/P-1 gene at the posterior end of planarians. Thus, various signaling molecules play an important role in planarian stem cell regulation. However, the molecular mechanisms directly involved in stem cell differentiation have remained unclear. Here, we demonstrate that one of the planarian LIM-homeobox genes, Djislet, is required for the differentiation of Djwnt1/P-1-expressing cells from stem cells at the posterior end. RNA interference (RNAi)-treated planarians of Djislet [Djislet(RNAi)] show a tail-less phenotype. Thus, we speculated that Djislet might be involved in activation of the Wnt signaling pathway in the posterior blastema. When we carefully examined the expression pattern of Djwnt1/P-1 by quantitative real-time PCR during posterior regeneration, we found two phases of Djwnt1/P-1 expression: the first phase was detected in the differentiated cells in the old tissue in the early stage of regeneration and then a second phase was observed in the cells derived from stem cells in the posterior blastema. Interestingly, Djislet is expressed in stem cell-derived DjPiwiA- and Djwnt1/P-1-expressing cells, and Djislet(RNAi) only perturbed the second phase. Thus, we propose that Djislet might act to trigger the differentiation of cells expressing Djwnt1/P-1 from stem cells.

KEY WORDS: Planarian, Regeneration, LIM-homeobox transcription factor, Islet, Wnt signaling

INTRODUCTION
LIM-homeobox gene family members play an important role as transcription factors in tissue-specific differentiation and body patterning during development in both vertebrates and invertebrates (Curtiss and Heilig, 1998; Kadras et al., 2004). The Islet family of LIM-homeobox transcription factors has been well conserved throughout evolution (Srivastava et al., 2010). In particular, Islet1 (Isl1) protein is the earliest marker for motor neuron differentiation (Karlsson et al., 1990; Ericson et al., 1995; Yamada et al., 1993; Thor and Thomas, 1997; Pfaff et al., 1996). However, Isl1 function is not restricted to developing neuronal structures, as Isl1 is also required for pituitary precursor cell proliferation (Takuma et al., 1998) and pancreas organogenesis (Ahlgren et al., 1997). Recent studies have demonstrated that Isl1 plays a key role in cardiac development and serves as a marker for pluripotent cardiovascular progenitors in several species, including mouse, rat, and human (Cai et al., 2003; Buckingham et al., 2005; Moretti et al., 2006; Kattmann et al., 2006; Sun et al., 2007). Therefore, Isl1 is required for the proliferation, survival and migration of progenitor cells or multipotent stem cells to form various organs.

Planarians are a superb model for studying the mechanisms of stem cell systems and regeneration because of their robust ability to regenerate themselves using pluripotent stem cells, called neoblasts (Baguñà et al., 1989; Agata and Watanabe, 1999; Reddien and Sánchez Alvarado, 2004; Agata et al., 2006). Planarians can regenerate all organs, including the central nervous system, gut and muscle within one week of amputation (Agata et al., 2003; Umesono and Agata, 2009). Previously, several marker genes of neoblasts were identified in the planarian Dugesia japonica (Shibata et al., 1999; Salvetti et al., 2000; Ogawa et al., 2002; Orii et al., 2005; Rossi et al., 2007; Yoshida-Kashikawa et al., 2007; Hayashi et al., 2010; Rouhana et al., 2010). However, the detailed mechanisms of how stem cells are regulated during regeneration remain unclear.

Recently, it was reported that canonical Wnt/β-catenin signaling is required for posterior specification and regeneration in planarians. RNA interference (RNAi) of Smed-βcatenin1 [Smed-βcatenin1(RNAi)], which is an activator of Wnt signaling, in the planarian species Schmidtea mediterranea leads to Janus-heads (an allusion to the Roman god Janus) formation in the tail region (Gurley et al., 2008; Petersen and Reddien, 2008; Iglesias et al., 2008). Comparing the knockdown of various posterior Wnt family genes, Smed-wntP-1(RNAi), which has the most posterior-specific expression, shows Janus-heads formation similar to Smed-βcatenin1(RNAi) (Adell et al., 2009; Petersen and Reddien, 2009). Smed-wnt11-2(RNAi), however, shows a tail formation defect and inappropriate midline patterning after posterior amputation (Gurley et al., 2010). Furthermore, it has been shown that Hedgehog (Hh) signaling is upstream of Wnt/β-catenin signaling and is involved in the establishment of anterior-posterior (AP) polarity (Rink et al., 2009; Yazawa et al., 2009). These studies clearly indicate that
signaling molecules plays a crucial role, not only in regeneration, but also in cell differentiation. The direct regulatory mechanism involved in neoblast differentiation, however, has remained unclear.

In this study, we focused on a LIM-homeobox transcription factor, *Dugesia japonica* *islet* (*Djislet*). Interestingly, *Djislet* (RNAi) shows a tail-less phenotype in addition to defects of the nervous system. Here, we describe a new insight into the relationship between Wnt signaling and a LIM homeobox transcription factor in the process of stem cell differentiation, and discuss how planarians coordinate the differentiated cells and stem cells to regulate regeneration.

Recently, unifying the nomenclature of the planarian Wnt family genes was proposed by a group using *S. mediterranea*: Smed-wntP-1, Smed-wnt2-1 and Smed-wnt2-2 were renamed Smed-wnt1, Smed-wnt2 and Smed-wnt11-5, respectively (Gurley et al., 2010). However, the nomenclature has not yet been well fixed. We had independently isolated two Wnt family genes from *Dugesia japonica* and named them *DjwntA* and *DjwntB* (Kobayashi et al., 2007). Recently, we also cloned other Wnt family genes from *Dugesia japonica* using GS FLX System (Roche 454). Here, we named or renamed the *D. japonica* Wnt genes according to a combination of new and old nomenclature as follows: *Djwnt1*-P-1 (previously *DjwntP-1*), *Djwnt2/B* (previously *DjwntB*), the homolog of *Smed-wnt2*), *Djwnt11-5*-P-2 (the homolog of *Smed-wnt11-5*), *Djwnt11-1* (the homolog of *Smed-wnt11-1*) and *Djwnt11-2* (unchanged).

**MATERIALS AND METHODS**

**Animals**

A clonal strain, sexualizing special planarian (SSP) (2n=16), of the planarian *Dugesia japonica* was used (Ito et al., 2001). Planarians were maintained in an asexual state in autoclaved tap water at 24°C. They were fed chicken liver every 2 weeks. Animals were starved for at least 1 week before experiments. Planarians with a body length of ~8 mm were used in all experiments. For regeneration studies, planarians were cut into three fragments (head, trunk and tail) by transverse amputation anterior and posterior to the pharynx. These fragments were allowed to regenerate and then fixed at specific times.

**γ-ray irradiation**

Animals were irradiated with 15 gray of γ-rays using a cesium source (Gammarcell 40 Exactor, Best Theratronics). Irradiated animals lost almost all regenerating ability and showed significantly decreased expression levels of stem cell marker genes. At least four days after irradiation, animals were amputated for regenerating studies.

**cDNA clones**

cDNA clones encoding the respective proteins *Djislet* (accession number AB610877), *Djyfrp-B* (AB610880) and *Djα-tubulin* (AB610878) were identified in a previously constructed library of expressed sequence tags (ESTs) (Mita et al., 2003). Partial cDNA fragments encoding *Djwnt1/P-1* (AB504744), *Djwnt11-5/P-2* (AB610882), *Djwnt11-1* (AB610881) and *Djwnt11-2* (AB504745) were cloned by PCR. The respective PCR primers were designed based on the sequences obtained using a GS FLX System (Roche 454). These cDNA fragments were cloned using the pCR2.1-TOPO vector (Invitrogen).

**Whole-mount in situ hybridization and immunohistochemistry**

Whole-mount in situ hybridization was performed with digoxigenin (DIG)-labeled riboprobes (Roche Diagnostics). They were prepared using PCR products from cDNA pBluescript SK(−) vectors containing the inserts from EST clones of interest, or pCR2.1-TOPO vectors newly cloned as described above. Animals were treated with 2% HCl in 5/8 Holtfreter’s solution for 5 minutes at 4°C and fixed in 5/8 Holtfreter’s solution containing 4% paraformaldehyde and 10% methanol for 30 minutes at room temperature. Hybridization and detection of DIG-labeled RNA probes were carried out as previously described (Umesono et al., 1997; Agata et al., 1998). In addition, all samples were processed with in situ chip (S) (ALOKA) for treatment of a large number of specimens.

Double staining for detection of mRNA expression was performed essentially as described (Yazawa et al., 2009). For the detection of DIG- or fluorescein-labeled RNA probes, samples were incubated with specific antibodies conjugated with alkaline phosphatase or horseradish peroxidase (1:2000; Roche Diagnostics). To develop fluorescent color, TSA kit No. 2 (Molecular Probes) and then an HNPP Fluorescent Detection Set (Roche Diagnostics) were used according to the respective manufacturer’s instructions. Cell nuclei were stained with Hoechst 33342. Before treating with an HNPP fluorescent detection set, appropriate samples were subjected to immunohistochemistry using anti-DjPiwiA monoclonal antibody (1:1000), anti-DjSYT (1:2000) and anti-α-tubulin Ab-2 (NeoMarkers, 1:200) as previously described (Yoshida-Kashikawa et al., 2007; Tazaki et al., 1999; Cebria and Newmark, 2005). In brief, specimens were incubated with the primary antibody overnight at 4°C and then with fluorescence-labeled secondary antibodies (Alexa Fluor 488 or 633; Molecular Probes, 1:500). Fluorescence was detected with a confocal laser scanning microscope FV1000 (Olympus).

**RNA interference (RNAi)**

Double-stranded RNA (dsRNA) was synthesized from in vitro transcription reactions with a MEGAscript RNAi Kit (Ambion), using PCR products with flanking 17 promoters from appropriate cDNA clones. Feeding RNAi was performed according to bacterial-feeding protocols (Reddien et al., 2005; Gurley et al., 2008) and a dsRNA-feeding protocol (Rouhana et al., 2010). Ten animals were each fed 13 μl of dsRNA-food, which was made up of 4 μg of dsRNA, 50% chicken liver paste and 0.2% agarose (Type IX, SIGMA), every 3 days for three feedings. Control animals were fed distilled water (DW); the solvent for dsRNA) instead of dsRNA. When we performed RNAi of Wnt genes (*Djwnt1/P-1*, *Djwnt11-5/P-2*, *Djwnt11-1* and *Djwnt11-2*), dsRNA was injected into planarians essentially as described (Sánchez Alvarado and Newmark, 1999). Control animals were injected with DW. For regeneration studies, planarians were cut 3 days after the last feeding or injection.

**Quantitative real-time PCR (qPCR) analysis**

In RNAi-regeneration studies, RNAi head fragments (n=10) were stump regions of the posterior end at the indicated regeneration time except in the γ-ray irradiation study in which we used whole head fragments (n=10). Total RNA was extracted from these stumps or whole regions using ISOGEN-LS (Nippon Gene), and cDNA was synthesized from 500 ng of total RNA using a QuantitiTeck Reverse Transcription Kit (Qiagen). qPCR analysis of gene expression levels was performed as previously described (Ogawa et al., 2002). The synthesized cDNAs were appropriately diluted (1/60), and then used for gene expression analyses by qPCR. Ten microliters of qPCR mixture including 1× Quantitect SYBR green PCR master mix (Qiagen), 0.3 μmol/l gene-specific primers and 3 μl of diluted cDNA template were analyzed using an ABI PRISM 7900 HT (Applied Biosystems). The reactions were carried out at 95°C for 2 minutes, 95°C for 15 minutes, 50 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 1 minute. PCR primers for each target gene are listed in Table S1 in the supplementary material. Measurements were performed in quadruplicate for technical replicates (coefficient of variation was 0.1-2.8% for all primer sets) and were normalized by the expression level of *Djα-tubulin*. The expression levels of target genes were expressed relative to the level in the control, which was taken as 1.0.

**Fluorescence-activated cell sorting (FACS)-based single-cell PCR**

Dissociation of planarian cells and staining with dyes were performed as previously described (Hayashi et al., 2006). Pieces of the posterior end from regenerating-head fragments (1 and 3 days after amputation) were collected from 200 fragments and dissociated into single cells. Flow cytometric analyses and collection of single cells for RT-PCR were carried out using a FACS Vantage SE triple-laser cell sorter (Becton Dickinson) as previously described (Hayashi et al., 2006; Hayashi et al., 2010). The PCR primers are listed in Table S2 in the supplementary material.
RESULTS

Djislet RNAi causes tail-less regeneration phenotype in planarians

We isolated a clone, encoding a LIM-homeodomain (LIM-HD) protein from the planarian EST library (see Fig. S1 in the supplementary material). The predicted amino acid sequence has two highly conserved LIM domains and a homeodomain (see Fig. S2 in the supplementary material), showing high similarity to the Islet of vertebrates. Thus, we named this gene Djislet. To explore the function of Djislet in planarians, we investigated expression patterns and the gene knockdown phenotype of Djislet. Djislet was expressed in the central nervous system (CNS), including the cephalic brain and ventral nerve cords (VNCs), and the marginal zone of the pharynx in intact animals. These expression patterns showed resistance to γ-ray irradiation, suggesting that Djislet was largely expressed in differentiated cells in intact animals (see Fig. S3 in the supplementary material).

In spite of such an expression pattern, Djislet(RNAi) showed a tail-less regeneration phenotype in both head and trunk fragment regenerants (Fig. 1B). To analyze the tail-less phenotype, we examined the structure of the posterior region using anti-α-tubulin antibody to visualize the axon bundles of the VNCs and the transverse commissures (Cebrià and Newmark, 2005). Interestingly, Djislet(RNAi) showed fusion of the VNCs (which normally separate) at the region posterior to the pharynx, similar to the Smed-wnt1-2(RNAi) phenotype previously described (Fig. 1C) (Adell et al., 2009; Gurley et al., 2010). These results suggest that Djislet functions in posterior regeneration and in acquisition of posterior structures. Hence, regenerants from the head pieces (‘head regenerants’) showed clearer phenotypes (tail-less but with formation of a pharynx) than those from trunk and tail pieces, and therefore we used head regenerants in the following experiments (except for Fig. 5E).

From these observations, we confirmed the tail-less phenotype at 15 days after amputation. Animals at the top are head regenerants and those at the bottom are trunk regenerants. Numbers represent the fraction of tested regenerants showing the indicated phenotype. Dashed lines indicate the amputation sites. (Fig. 1A) shows the schematic of the RNAi-regeneration analysis. Animals treated with RNAi were cut transversely into three fragments: head (H), trunk (TR) and tail. (B) Analysis of the Djislet(RNAi) phenotype at 15 days after amputation. Animals at the top are head regenerants and those at the bottom are trunk regenerants. Numbers represent the fraction of tested regenerants showing the indicated phenotype. (C) Whole-mount immunostaining with anti-α-tubulin at the tail region in the head regenerants at 14 days. The nuclear staining is shown by Hoechst (blue). Boxed area of the schematic in the left panel indicates the site for confocal imaging. ph, pharynx; v, ventral nerve cord. Scale bars: 200 μm. (D) Relative gene expression analysis of Djislet(RNAi) animals by qPCR in the posterior region (the boxed area in the diagram) of the head regenerants at 10 days. On the left is a schematic of the expression sites of the genes along the anterior-posterior axis, which are indicated as horizontal bars for each gene. On the right, the expression levels of target genes are shown relative to the level in control head regenerants (taken as 1.0). *P<0.01, ***P<0.0001 versus control (gray bars).

The tail-less phenotype caused by Djislet(RNAi) is related to Djwnt1/P-1 function

Because Djislet(RNAi) showed a tail-less phenotype, we speculated that this phenotype might be related to Wnt signaling. In S. mediterranea, Smed-wnt1 was expressed most posteriorly among the posterior Wnt genes, and only Smed-wnt1(RNAi) animals showed Janus-heads formation in addition to the tail-less phenotype (Adell et al., 2009; Petersen and Reddien, 2009). Thus, Smed-wnt1 is speculated to be a candidate for the most upstream gene for posteriorization among posterior Wnt family genes. Interestingly, we observed that Djislet and Djwnt1/P-1 were expressed in the dorsal-midline of the posterior blastema at day 3 with the same pattern (Fig. 2A,B, arrowhead). Furthermore, the expression of Djwnt1/P-1 in the dorsal-midline was eliminated after Djislet(RNAi) (Fig. 2B, center). By contrast, Djwnt1/P-1(RNAi) animals showed overexpression of Djislet in
the posterior blastema (Fig. 2A, right). This overexpression seemed to be a secondary effect, caused by anteriorization after Djwnt1/P-1(RNAi). Thus, the tail-less phenotype caused by Djislet(RNAi) might be related to the ablation of Djwnt1/P-1 expression in the posterior blastema.

The tail-less phenotype caused by Djislet(RNAi) is different from the Djwnt1/P-1(RNAi) phenotype at a later stage

We did, however, find a difference between the Djislet(RNAi) and Djwnt1/P-1(RNAi) phenotypes. Although both phenotypes showed an inhibition of posterior regeneration, at a later stage of posterior regeneration, Djwnt1/P-1(RNAi) showed Janus-heads formation in addition to some tail-less regenerants (Fig. 3A). By contrast, Djislet(RNAi) did not show Janus-heads formation at all (Fig. 1B, Fig. 3A). Staining of the regenerants for expression of Dj frizzled-T (DjfzT), a posterior-specific Wnt receptor gene (Yazawa et al., 2009), showed that there was a dramatic reduction of the tissue expressing DjfzT in both Djislet(RNAi) and Djwnt1/P-1(RNAi) (Fig. 3B). This implies that both RNAi phenotypes showed a defect in the tail region. The Djislet(RNAi) animals, however, always formed a pharynx, like the control, whereas the Djwnt1/P-1(RNAi) animals did not (Fig. 3B'). Therefore, we decided to classify the two tail-less phenotypes caused by Djwnt1/P-1(RNAi) and Djislet(RNAi), as pharynx/tail-less (P/T-less) and tail-less (T-less) phenotype, respectively. To address what caused this difference, we examined the expression pattern of Djwnt1/P-1 in the early stages of regeneration. The expression pattern of Djwnt1/P-1 at day 1 showed a different modality compared with that at day 3. In contrast to day 3, the head fragments displayed a dot-like pattern on the ventral side of the posterior end at day 1, as previously reported in S. mediterranea (Fig. 3C) (Petersen and Reddien, 2009; Gurley et al., 2010). Interestingly, this dot-like expression pattern was not affected by Djislet(RNAi) (Fig. 3C). These results suggest that the difference between Djislet(RNAi) and Djwnt1/P-1(RNAi) phenotype was determined by whether or not Djwnt1/P-1 was expressed in the early stages of regeneration. Thus, we propose that the phenotypes of Janus-heads and P/T-less were caused by ablation of the Djislet(RNAi) animals at day 1, whereas the Djwnt1/P-1(RNAi) animals did not show this pattern at day 1.

Fig. 3. Djislet(RNAi) phenotype is different from Djwnt1/P-1(RNAi). (A) Live images of Djislet(RNAi) and Djwnt1/P-1(RNAi) animals obtained from the head regenerants (H) at day 9. (B) Expression pattern of DjfzT (fzT), a posterior-specific marker gene, in the head regenerants at day 10 by whole-mount in situ hybridization (dorsal view). (B') Confocal images of boxed areas in B. Nuclei are stained with Hoechst (blue). Asterisks indicate the newly developed pharynx. Note that the pharynxes of the control and Djislet(RNAi) animals are shown by the elliptical borderline (arrowhead) and the Hoechst signal of these central regions (left/center). Djwnt1/P-1(RNAi) animals did not show this region (right). (C) The expression pattern of Djwnt1/P-1 in the posterior end of head regenerants (indicated in the diagram) at day 1 (ventral view). Dotted line indicates the dot-like pattern of Djwnt1/P-1 expression in the stump region of the ventral side. Dashed lines indicate the amputation site. Arrows indicate ectopic eyes. Numbers represent the fraction of tested regenerants showing the indicated phenotype. Scale bars: 200 μm.
Islet regulates Wnt signaling

Djislet is required for the second phase of Djwnt1/P-1 expression

Previous studies demonstrated that the expression of Smed-wnt1 has two phases: an γ-ray-insensitive wound-induced expression (the dot-like pattern) phase and an γ-ray-sensitive expression (the concentrated pattern) phase in S. mediterranea (Petersen and Reddien, 2009; Gurley et al., 2010). Therefore, we examined the gene expression patterns and expression levels of Djwnt1/P-1 in Djislet(RNAi) animals during posterior regeneration by in situ hybridization and qPCR analysis (Fig. 4). We observed two phases of Djwnt1/P-1 expression conforming closely to the expression patterns of Smed-wnt1 seen by in situ hybridization (Fig. 4A). Moreover, the concentrated pattern was remarkably reduced by Djislet(RNAi), whereas the dot-like expression pattern was not affected (Fig. 4A).

Next, we validated these results quantitatively by qPCR analysis, because previous studies did not quantitatively analyze the wnt1/P-1 expression levels during posterior regeneration. We found that the expression level of Djwnt1/P-1 showed two clear waves: the first at day 1 and the second at day 3 (Fig. 4B, black line). These waves were designated the first phase of wnt1/P-1 expression and the second phase of wnt1/P-1 expression, respectively (Fig. 4, green and pink band). To investigate which cell types contribute to these expression patterns, we identified cell types by γ-ray sensitivity. In planarians, stem cells can be eliminated within at least 4 days of γ-ray irradiation (Shibata et al., 1999; Hayashi et al., 2006). We also investigated the expression levels of Djwnt1/P-1 in Djislet(RNAi) animals and γ-ray-irradiated animals. Both γ-ray-irradiated and Djislet(RNAi) animals clearly showed ablation only of the second phase of wnt1/P-1 expression, but not the first phase, in agreement with in situ hybridization analysis (Fig. 4B, blue and red lines). Consequently, we speculated that these two phases of wnt1/P-1 expression can be classified into islet-independent and islet-dependent phases, respectively.

Djislet functions in the stem cell-derived cells expressing Djwnt1/P-1

Because Djislet regulates the second (γ-ray sensitive) phase of wnt1/P-1 expression, we speculate that Djislet functions in the stem cell-derived cells. To address this matter, we examined the expression pattern of Djislet in both the first phase (day 1) and second phase (day 3), with and without γ-ray irradiation (Fig. 5A-D). Djislet expression showed a similar pattern to Djwnt1/P-1 expression (dot-like pattern) in control animals at day 1 (Fig. 5A,B, dotted lines) as well as the dorsal-midline pattern at day 3 (Fig. 2, Fig. 5A,B, arrowheads). However, in γ-ray-irradiated animals, the expression of Djislet in the posterior end of regenerants was completely eliminated, in contrast to the first phase of wnt1/P-1 expression (Fig. 5C,D). We thus confirmed that Djislet-expressing cells in the posterior region are γ-ray-sensitive cells.

Next, we investigated the co-expression of Djislet and Djwnt1/P-1 using dual fluorescence in situ hybridization and confocal imaging (Fig. 5E). In the head regenerants, as expected, we could see a few cells that co-expressed both genes at the tip of the posterior blastema on the dorsal side at day 3, although we did not find cells that co-expressed both Djwnt1/P-1 and Djislet on the ventral side at day 1 (see Fig. S6 in the supplementary material). Then we performed dual in situ hybridization combined with DpiwiA immunostaining, because DpiwiA is known to be a stem cell and stem cell-derived cell marker (Fig. 5E) (Yoshida-Kashikawa et al., 2007). We found a few triple-positive cells (Djwnt1/P-1, Djislet and DpiwiA protein) in the proximal region of the posterior blastema of the trunk regenerants at day 3 (Fig. 5E,E’, arrowhead). Because the expression levels of DpiwiA protein in the triple-positive cells were relatively weaker than those of undifferentiated stem cells (Fig. 5E, anti-DpiwiA, upper side of panel), these triple-positive cells were likely to be stem cell-derived cells (see Discussion). Moreover, the expression level of DpiwiA protein gradually decreased from the triple-positive cells towards
the tip of the posterior blastema, along with Djislet and Djwnt1/P-1 double-positive cells (Fig. 5E/H11032, dotted lines). The posterior-most Djwnt1/P-1-expressing cells did not express Djislet (Fig. 5E/H11032, asterisks). Given the gradually decreasing expression of DjPiwiA protein, the posterior-most cells might be terminally differentiated cells. The overlapping expression of Djislet and Djwnt1/P-1 raises the possibility that Djislet functions to regulate only the second phase of Djwnt1/P-1 expression, which is activated in stem cell-derived cells in the posterior end of the regenerants.

Djislet regulates the posterior genes in the γ-ray-sensitive posterior blastema cells

To understand the relationship between Djislet and other posterior-specific Wnt and Hox genes, we examined the effect of Djislet(RNAi) on the expression of posterior genes. In contrast to the significant reduction of Djwnt1/P-1 expression in Djislet(RNAi) animals from day 2 (Fig. 4), no major difference was observed in the expression of Djwnt11-1, Djwnt11-2 or DjAbd-Ba between control and Djislet(RNAi) animals in the posterior blastema at day 2 (Fig. 6A-C). However, at day 3, the Djislet(RNAi) animals had lost the expression of these genes (Fig. 6A-C). By contrast, the expression of Djwnt11-5/P-2 was not affected by Djislet(RNAi) in the posterior blastema (Fig. 6D).

We examined the gene expression level of posterior genes during regeneration in the posterior blastema treated with Djislet(RNAi) and γ-ray irradiation (Fig. 6E). The seven posterior genes were categorized into three classes: the class I gene is not directly under the regulation of Djislet and is expressed mainly in γ-ray-insensitive cells (Djwnt11-5/P-2), class II genes are partly downregulated in Djislet(RNAi) and expressed in both γ-ray-sensitive and -insensitive cells (Djwnt11-1, DjfzT, plox4-Dj and plox5-Dj), class III genes are strongly downregulated in Djislet(RNAi) and expressed mainly in γ-ray-sensitive cells (Djwnt11-2 and DjAbd-Ba). The genes of classes II and III showed no difference in expression level between control and Djislet(RNAi) animals until day 2 [Fig. 6E, no significant change in Djislet(RNAi) vs control], but then showed clearly decreased expression by Djislet(RNAi) at day 3 [Fig. 6E, #: Djislet(RNAi) at day 3 vs Djislet(RNAi) at day 2]. Moreover, upon irradiation, the expression of genes in class III was barely detected (Fig. 6E, no significant change in γ-ray vs γ-ray at day 0) and genes in class II showed increased but still lower expression levels compared with control (Fig. 6E, *: γ-ray vs γ-ray at day 0, *: γ-ray vs control). A previous study reported that expression of both Smed-wnt11-1 and Smed-wnt11-2 is absent in irradiated head regenerants (Gurley et al., 2010). The difference in results of
wnt11-1 expression between that study and our own might be caused by the difference of the sensitivity of gene expression between qPCR and in situ hybridization analysis. By contrast, the expression levels of Djwnt11-5/P-2 (class I) remained unchanged from the control until day 2 (Fig. 6E, no significant change or γ-ray vs control), and showed no significant change or a slight increase from day 2 to day 3 in Djislet(RNAi) and irradiated animals (Fig. 6E). This finding in irradiated animals was in close accord with the reported features of Smed-wnt11-5 (Petersen and Reddien, 2009; Gurley et al., 2010). These results suggest that Djislet is required for the maintenance and upregulation of the posterior genes expressed in the γ-ray-sensitive posterior blastema cells.

**Djwnt1P-1 signal is upstream of the posterior Wnt genes**

To investigate epistasis in these four posterior Wnt genes (Fig. S4 in the supplementary material), we examined their gene expression levels by qPCR in the posterior blastema in animals with RNAi for each Wnt gene and Djβ-cateninB (Fig. 6F). Djwnt1P-1(RNAi) animals exhibited a decrease in the expression levels of the other Wnt and posterior genes at day 1 and day 3, as did Djβ-cateninB(RNAi) animals (Fig. 6F). Moreover, Djwnt1P-1(RNAi) did not show an increase in the expression of the anterior genes at day 1, although it showed a slight decrease in the expression of the posterior genes (Fig. 6F; see Fig. S9 in the supplementary material) and this decrease was not influenced by anteriorization. In addition, Djβ-cateninB(RNAi) had little effect on the expression of Djwnt1P-1 at day 1, as previously described (Petersen and Reddien, 2009; Rink et al., 2009). This result showed that, at least in the first phase, Djwnt1P-1 is expressed independently of the β-catenin pathway, in contrast to the other Wnt and posterior genes. By contrast, RNAi knockdown of the Wnt genes Djwnt11-1, Djwnt11-2 and Djwnt11-5/P-2 and Djβ-cateninB at day 1 (upper) and at day 3 (lower) after amputation. Note that the expression level of Djislet was not reduced in the knockdown animals of any of the genes, and was rather increased by anteriorization of their posterior end in Djwnt1P-1(RNAi) or Djβ-cateninB(RNAi) at day 3. The expression levels were normalized and are shown relative to the level in the posterior end of the control (taken as 1.0). ND, not detected.

**DISCUSSION**

**Islet functions in the Wnt-expressing cells of planarian**

The relationship between LIM-homeobox transcription factors and Wnt signaling has been reported in the developmental processes of several organisms (Riddle et al., 1995; Adams et al., 2000; Matsunaga et al., 2002; O’Hara et al., 2005). Furthermore, β-catenin is involved in Isl1 expression in cardiac progenitors in the mouse embryo (Lin et al., 2007; Kwon et al., 2009). However, the Islet family homologs have not been reported to regulate Wnt
Only the second phase of \textit{wnt1/P-1} expression is affected by \textit{Djislet}

\textit{Djwnt1/P-1} expression has two phases, the first phase and the second phase, during posterior regeneration. These two phases of \textit{wnt1/P-1} expression have been described previously (Petersen and Reddien, 2009; Gurley et al., 2010). However, the detailed mechanism of the regulation of posterior regeneration via two phases of \textit{wnt1/P-1} expression has not been elucidated because the separation of these two phases has been difficult. However, we have succeeded in segregating these two phases using \textit{Djislet} (RNAi). We found that \textit{Djislet} is expressed only in the second phase of \textit{wnt1/P-1}-expressing cells (Fig. 5; see Fig. S6 in the supplementary material) and regulates only the second-phase of \textit{wnt1/P-1} expression (Fig. 4). We speculate that the first phase expression in differentiated cells is required for recruiting stem cells into \textit{Djwnt1/P-1}-expressing cells, and then a kind of positive circuit of Wnt-signaling might be established. \textit{Djislet} is required for differentiation of the \textit{Djwnt1/P-1}-expressing cells from the Piwi-positive stem cells. Here, we propose one possible model of the sequential posterior regeneration process divided into two phases based on \textit{Djislet} function (Fig. 7).

The activity of the islet-independent first phase of \textit{wnt1/P-1} expression

In the early stage of posterior regeneration (day 1), the first phase of \textit{wnt1/P-1} expression is upregulated in the differentiated cells at the posterior end independently of \textit{Djislet} function (Fig. 4). Recently, it was demonstrated that this early expression of \textit{wnt1/P-1} is under the control of Hh signal activity, and that elimination of \textit{wnt1/P-1} function caused by inhibition of Hh signaling induces anteriorization of the posterior end (Rink et al., 2009; Yazawa et al., 2009).

Interestingly, absence of the first phase of \textit{wnt1/P-1} expression caused anteriorization (Janus-heads and P/T-less phenotype). Moreover, expression of \textit{Djwnt11-5}/P-2 occurred in \gamma-ray-irradiated head regenerants, indicating that posterior fate is determined independently of stem cells (Fig. 6) (Petersen and Reddien, 2009; Gurley et al., 2010). Thus, we propose that the first phase of \textit{wnt1/P-1} is involved in the decision of posterior specification through the activation of the expression of posterior genes (class I, class II and an unidentified factor) via the \beta-catenin pathway in the differentiated cells (Figs 6, 7). In support of this model, the synergy between \textit{Smed-wnt1} and \textit{Smed-wnt11-5} for the decision of posterior specification has been reported (Petersen and Reddien, 2009). In addition, \textit{Smed-wnt1} (RNAi) animals that were P/T-less had smaller than normal posterior blastemas (Adell et al., 2009). By contrast, \textit{Djislet} (RNAi) animals showed posterior blastema formation with a transient increase in the expression of posterior genes (Fig. 6). We speculate that the first phase of \textit{wnt1/P-1} signaling might be involved in blastema formation accompanying the activation of the posterior genes in stem-cell-derived cells (Fig. 7).

The activity of the islet-dependent second phase of \textit{wnt1/P-1} expression

In the middle stage of posterior regeneration (day 3), the second phase of \textit{wnt1/P-1} expression emerges in place of the first phase (Fig. 4), i.e. the posteriorization signal from the differentiated cells is inherited by stem-cell-derived cells in the tip of the posterior blastema. At present, the mechanism of this inheriting is unclear. Perhaps \beta-catenin and some additional signal molecules are needed to generate the second phase of \textit{wnt1/P-1}-expressing cells (Fig. 6F) (Petersen and Reddien, 2009). However, our results clearly demonstrate that when the \textit{Djislet} function is absent, the posteriorization signal cannot be maintained in the blastema (Fig. 6).
As a result, these animals show a T-less phenotype (Fig. 1). Moreover, *Djwnt11-P-2* (class I) seems not to be expressed under control of *Djislet* expression (Fig. 6). *Djwnt11-P-2* is expressed mainly in the differentiated cells (Fig. 6E) (Petersen and Reddien, 2009; Gurley et al., 2010), and is also expressed in the pharyngeal region. By contrast, the other posterior genes (class II and III) show tail-specific expression (see Fig. S4 in the supplementary material) (Orii et al., 1999; Nogi and Watanabe, 2001; Yazawa et al., 2009). Hence, we propose that *Djislet* has distinct local effects on the stem cell-derived posterior blastema cells that form the tail structure via tail-specific-posterior genes (class II, III and unidentified factor(s); Figs 6, 7). Furthermore, it is known that *Smed-wnt1*-expressing cells are rapidly turned over in intact animals (Petersen and Reddien, 2009; Gurley et al., 2010). We also found that *Djislet* was co-expressed with *Djwnt1-P-1* in the γ-ray-sensitive cells in intact animals and appeared to regulate *Djwnt1-P-1* expression in these cells (see Fig. S10 in the supplementary material). However, the morphological phenotype does not appear in intact animals after *Djislet* RNAi (see Fig. S11 in the supplementary material). These results raise the possibility that *islet*-dependent *wnt1*-P-1 expression is not related to posterior specification in either homeostasis or regeneration. A recent study showed that *Smed-wnt11-2*(RNAi) results in the posterior VNCs converging and fusing at the midline (Adell et al., 2009; Gurley et al., 2010). Our results also indicated that *Djislet*(RNAi) and partial RNAi of *Djwnt1-P-1* animals showed the fusion of VNCs (Fig. 1C; see Fig. S7 in the supplementary material), suggesting that this might be a secondary action of *Djwnt11-2* defects caused by ablation of the second phase of *wnt1*-P-1 signaling. Actually, *Djwnt11-2* expression was remarkably downregulated by *Djislet*(RNAi) or *Djwnt1-P-1*(RNAi) (Fig. 6E,F). Thus, it seems that *islet*-dependent *wnt1*-P-1 signaling mediates proper tail formation through the function of posterior genes.

Partial RNAi of *Djwnt1-P-1* induced a T-less phenotype similarly to *Djislet*(RNAi) (see Figs S7, S8 in the supplementary material). This result is also consistent with the possibility that the T-less phenotype is a weak anteriorization phenotype, although the animals did not ectopically express anterior-specific genes (Fig. 1D; see Fig. S8 in the supplementary material). Therefore, we have to leave open the alternative possibility that the tail defects caused by *Djislet*(RNAi) might be a secondary consequence of a weak anteriorization of the axis. However, when we carefully quantified the expression level of *Djwnt1-P-1* after partial RNAi of *Djwnt1-P-1* by qPCR, we found that the first phase of *wnt1*-P-1 expression in differentiated cells was only slightly reduced (11%, not statistically significant) in the partial RNAi animals, although ~36% reduction (P<0.001) of the second-phase expression was observed (see Fig. S7D in the supplementary material). Thus, we suppose that partial RNAi of *Djwnt1-P-1* showed a similar phenotype to T-less of *Djislet*(RNAi) owing to retention of the first phase of *wnt1*-P-1 expression, and that the RNAi effect was different between differentiated cells and stem cell-derived cells. To address this issue precisely, it will be necessary to examine the function of *Djislet* in transcriptional control more directly using various genomic and proteomic approaches.

Collectively, our findings demonstrate that posterior regeneration occurs by coordination of the above-mentioned two modes of action of *Djwnt1-P-1* activity. *Djislet* seems to be one of the crucial factors regulating this coordinating mechanism through its control of the differentiation of *Djwnt1-P-1*-secreting cells from stem cells. We believe that the *Djislet* knockdown model proposed here provides new insights into the mechanism of regeneration and tissue repair by pluripotent stem cells.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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