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Molecular cloning and expression pattern of the *DjStag* gene in the planarian *Dugesia japonica* during embryonic development

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ABSTRACT. We examined STAG-related gene (*DjStag*) expression in the planarian *Dugesia japonica*. This species is common in Far Eastern countries. The *DjStag* cDNA includes 1362 bp and contains a 489-bp open reading frame corresponding to a deduced protein of 162 amino acids, with a 170-bp 5'-UTR and a 703-bp 3'-UTR. Phylogenetic analysis showed that *DjStag* is an STAG/STAG-like member. We examined the expression pattern of *DjStag* in this planarian during embryonic development by whole-mount *in situ* hybridization. *DjStag* was detected in embryonic cells in the germ band at early embryo stages. The number of *DjStag*-positive embryonic cells increased in stage 5. Later, it was mainly expressed in lateral region parenchyma. In juveniles, extensive expression of *DjStag* was observed not only in the head and tail regions, but also in the parenchyma between the epidermis and the gastrodermis. We conclude that *DjStag* is expressed in the cellular subset that will become the neoblast cells of the adult flatworm. *DjStag* may play an essential role in spatial and temporal regulation during planarian embryonic development.

Key words: Planarian; *DjStag*; Gene expression; Embryogenesis

INTRODUCTION

In eukaryotes, sister chromatids remain physically connected by cohesion from the time of their synthesis in S phase until they are separated in anaphase (Nasmyth et al., 2000; Sumara et al., 2000). Sister chromatid cohesion is a fundamental aspect of chromosome behavior that ensures the accurate inheritance of the genetic information in mitosis and meiosis (Lee and Orr-Weaver, 2001; Nasmyth, 2001; Hirano, 2002). Sister chromatid cohesion depends on cohesin, a conserved protein complex composed of a heterodimeric pair of structural maintenance of chromosome subunits, Smc1 and Smc3, and two sister chromatid cohesion proteins Scc1 and Scc3 (Michaelis et al., 1997; Losada et al., 1998; Sumara et al., 2000). In humans, hoptoad and other higher eukaryotes, two cohesin isoforms exist, each containing the subunits Smc1, Smc3, Scc1, and either one of the Scc3 orthologs STAG1/SA1 or STAG2/SA2 (Losada et al., 2000; Sumara et al., 2000; Prieto et al., 2001).

Previous study on cohesins has been mainly focused on chromatid cohesion and segregation; certain cohesins have been implicated in different cellular processes (Lara-Pezzi et al., 2004). Cohesin is essential not only for chromosome segregation but also double-strand break repair (Ström et al., 2004, 2007; Rubio et al., 2008). Cohesin is also implicated in gene-regulatory functions (Rubio et al., 2008). The possible function of STAG is not completely understood. Lara-Pezzi et al. (2004) provided evidence suggesting that STAG functions as a transcriptional co-activator by a mechanism involving protein-protein interactions with transcription factors.

Planarians have the ability to regenerate their whole body from any small amputated fragment of their body. Thus, they are excellent models for studying embryonic development and the biology of regeneration (Reddien and Sánchez Alvarado, 2004; Agata et al., 2007; Solana and Romero, 2009). In this study, we isolated and characterized the cDNA sequences of an STAG-related gene (*DjStag*) from the planarian *Dugesia japonica* and examined its expression pattern during embryonic development by whole-mount *in situ* hybridization.

MATERIAL AND METHODS

Animals and egg capsules

The planarians and egg capsules used belong to the race of *D. japonica* collected from a fountain in Quanhetou, Boshan, China. The animals were kept in autoclaved tap water at 20°C. The embryos were fixed and dissected as previously described (Solana and Romero, 2009). After proper dehydration, embryos could be stored in Eppendorf tubes containing 70% ethanol at -20°C until used. Juvenile planarians were collected every day and treated with 2% HCl for 5 min on ice, then fixed on ice for 3 h in Carnoy's fixative (ethanol:chloroform:acetic acid, 6:3:1) and stored in methanol (Palakodeti et al., 2008).

Cloning and sequence analysis of cDNA

The cDNA library of adult planarians was constructed with the Creator™ SMART™ cDNA Library Construction kit (Clontech) according to the method previously described (Liu et al., 2002). In a large-scale sequencing of the cDNA library, more than 1000 clones were

analyzed for coding probability with the DNATools program. The *DjStag* cDNA was derived from the planarian cDNA library. Comparison against the GenBank protein database was performed using the BLAST network server at the National Center for Biotechnology Information (NCBI). Phylogenic tree analysis was carried out using the PHYLIP 3.5c software package.

Northern blots

Northern blotting was carried out as previously described (Palakodeti et al., 2006; Wang et al., 2008). Total RNAs were extracted using the RNAiso Reagent (TaKaRa) according to the manufacturer protocol. The blots were hybridized with DIG-labeled antisense RNA probes at 48°C for 16 h. The signal was developed using nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3'-indolyl phosphate p-toluidine salt (BCIP).

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed essentially as previously described (Reddien et al., 2007; Palakodeti et al., 2008). Hybridization was performed at 50°C for 36 h with DIG-conjugated riboprobes in hybridization solution. The color was developed using NBT and BCIP, and the reaction was stopped by two rinses in PBS for 5 min. After bleaching in 5% H_2O_2 in methanol at room temperature under light, whole-mount *in situ* hybridizations of juveniles were carried out as in dissected embryos. For histological sectioning, some preparations of embryos and juveniles were embedded in paraffin wax and sectioned at 8 μm thickness. Whole-mount animals were observed with a Nikon SMZ1500 stereomicroscope and sections were observed with a Zeiss Axioskop-40 microscope.

RESULTS AND DISCUSSION

Sequence analysis of *DjStag*

The cDNA clone (GenBank accession No. GQ503880) obtained from the cDNA library of *D. japonica* is 1362 bp long, and its longest open reading frame, consisting of 489 bp, codes for a deduced protein of 162 amino acids with a predicted molecular mass of approximately 18.08 kDa. Its 5'-untranslated region (UTR) is 170 bp in length and the 3'-UTR is 703 bp long with a polyadenylation signal AATAAA and a poly (A) tail (Figure 1). The start codon (ATG) is flanked by a purine base at positions -3 and +4, matching the Kozak consensus sequence (Kozak, 1987), and is suggested to optimize translational efficiency. Therefore, the cDNA encodes a full-length protein.

Initial BLASTP search at NCBI revealed that the protein encoded by the planarian cDNA shared 49 (80/163), 39 (59/151), 37 (60/158), and 37% (60/158), identity to STAG/STAG-like protein of the schistosome, *Schistosoma mansoni* (GenBank accession No. CAZ30922); drosophila, *Drosophila melanogaster* (CAA74654); cow, *Bos taurus* (XP_001249706); human, *Homo sapiens* (NP_001036214), respectively. This similarity of the primary amino acid sequence suggested that the gene was a planarian STAG-like gene. Therefore, the cDNA appeared to code for a planarian STAG-like protein and was designated *DjStag* in this paper.

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GGA AGA TGA TGC TGA CGA AGA TGA AAA CGT TAA AAT CGA AGA ATT ACA TAA TCG TCG TAC     62
                                                                                  20

AAA TTT AGC TAT GTT TTT AAA TTG ATT GTT TAC AAC GTA TTA CCG ATA AAA GCT GCA AGC    122
                                                                                  40

CAC ATA TAT AAG TTT TAT TTG CGA TTT TTC AAT GAT TTT GGA GAT ATA ATG AAA GCT GGA    182
                                                                M   K   A   G     60

CTA GCT AAA GCG CGT GAA ATA AAC CGC TTA CAT ACC GCC AAA ATG GTT ATG GAG TGT TTA    242
L   A   K   A   R   E   I   N   R   L   H   T   A   K   M   V   M   E   C   L     80

ATA TCT GGC CTC AAC GAA TTG ATG GTT GAA CAA TCC GGA TTT GTT GAT AGG TCT ACT GAA    302
I   S   G   L   N   E   L   M   V   E   Q   S   G   F   V   D   R   S   T   E    100

GGA TTT CAA TCT CTT AAG GGA TTG GCG AGG CGT TTG AAT CTC AGT TTC GGT TTA GAT TTA    362
G   F   Q   S   L   K   G   L   A   R   R   L   N   L   S   F   G   L   D   L    120

ATG AAA ATA AGA GAA GCA ATG ATG GAA TTA CAT AAA ATG GCG ATA GAC ACA GTT CCC AAC    422
M   K   I   R   E   A   M   M   E   L   H   K   M   A   I   D   T   V   P   N    140

GCA ATG GTT GTT ACC GGA CCT TCT CGA CCT CCT GCT AAT TTG CTT TGT TTG GAA TTA GCA    482
A   M   V   V   T   G   P   S   R   P   P   A   N   L   L   C   L   E   L   A    160

GCT GAA TTC AGT AAT AAA CTC ATT GGT TCC GAT AAA AAA TTC ATA TTG GAT AGT ATT AAT    542
A   E   F   S   N   K   L   I   G   S   D   K   K   F   I   L   D   S   I   N    180

AAA ACA TTT CCT AAT CCT GGT GAA AAT GAG GGT TGG ATG TCA CTT TAC ACC TAT CGA ATG    602
K   T   F   P   N   P   G   E   N   E   G   W   M   S   L   Y   T   Y   R   M    200

AGC CTC GAC CCT GAC ACT ATG GAC AAC ACA AGT ACT CAC AAT GAA AAG TTG TCA TAG TGG    662
S   L   D   P   D   T   M   D   N   T   S   T   H   N   E   K   L   S   *        220

TAC AGG TTC AGC TCA TAA TAT AAT TAC TCC TCA ACC TCG TCC AAG AGG TCG ACC AAA AAA    722
                                                                                 240

TTA AAA GGT CCA ACT TCC ATT CAA CGT CCT CCG ATT CAC AGT ACT GCT ATC AAA AAA CGA    782
                                                                                 260

AAA TAC AAT CAC TCA ATA AAT TCA GTC TCC GTC GCA CAT CCT TCC ATA AAT GTT TCT TCG    842
                                                                                 280

ATA GAC GGC CCT AAT ATG CAT GAA GAA CCA TCT GCT CAA TCA ATT CCT CGA GTA TCT ATT    902
                                                                                 300

CGT GGA AGA ACA TTG AAA AAC AGA ATC AGT CTA TCT TTA TCA GAT CAG GCT TTT GAT AGC    962
                                                                                 320

CAA TAA TCC CAG ATT TTT CCT AAA TGT GTT GAT TGA GAC CAA TCG CAC AGA AGA TTT CAA   1022
                                                                                 340

ATT TAT TAT GTG TAG CAG CTT CCG TAT ATT TAT ATA TAT TTA ATA ATG AAG TTA TTT GTG   1082
                                                                                 360

ATG ATG ATG GTA TCG TTA CAC CAC TTG TGT TGA GGA GAT TAC GTG TCT TAT TGT TTG TTA   1142
                                                                                 380

TCC GAG TTC TTG TAA ATA TTC AAT TTG TTT ATA CAT AAA TAT TTC GTA TTT TCA TTA TAT   1202
                                                                                 400

TAT GAT AAA ACT CAA ATG CTA TCT TTG AAA TCA GCC AGC CTC CAG ATT CTT TGT ACA ATA   1262
                                                                                 420

AAC CTA TAT ATG TAC AAA AAT ATA ATT AGA TTA TTT TTG CTG TTT GAG CCT TGA AAT AAA   1322
                                                                                 440

TCT GGT TGA GCA AAA AAA AAA AAA AAA AAA AAA AAA AAA A                             1362
                                                                                 460
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Figure 1. Nucleotide and deduced amino acid sequences of planarian (*Dugesia japonica*) *DjStag* cDNA (GenBank accession No. GQ503880). The start codon is underlined. The asterisk represents the stop codon. The polyadenylation signal is boxed. The numbering of the nucleotide and amino acid sequences is shown to the right.

To shed light on the evolutionary position of *DjStag*, a phylogenetic tree was constructed using the amino acid sequence of *DjStag* and other representative members of the STAG/STAG-like proteins by a neighbor-joining method, along with *Homo sapiens* STAG as the outgroup (Figure 2). The tree indicated that *DjStag* is intermediate between insect STAG and amphibian STAG, forming a group with schistosome STAG.

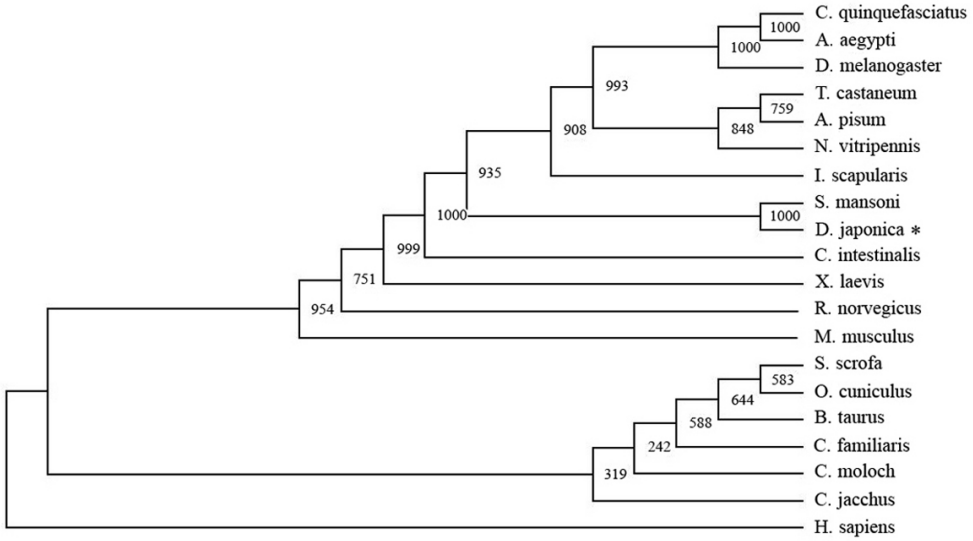

Figure 2. Phylogenic tree of STAG proteins constructed by the neighbor-joining method within the package PHYLIP 3.5c software package. *Homo sapiens* STAG was used as outgroup. The numbers refer to bootstrap values of 1000 replicates. GenBank accession numbers of the sequences used are as follows: *Homo sapiens*, NP_001036214; *Callithrix jacchus*, ABY90126; *Callicebus moloch*, ACA57925; *Canis familiaris*, XP_549232; *Bos taurus*, XP_001249706; *Oryctolagus cuniculus*, ACJ76606; *Sus scrofa*, XP_001926026; *Mus musculus*, CAM20006; *Rattus norvegicus*, XP_233108; *Xenopus laevis*, NP_001080997; *Ciona intestinalis*, XP_002121258; *Schistosoma mansoni*, CAZ30922; *Ixodes scapularis*, EEC02719; *Nasonia vitripennis*, XP_001600957; *Acyrthosiphon pisum*, XP_001950748; *Tribolium castaneum*, XP_966898; *Drosophila melanogaster*, CAA74654; *Aedes aegypti*, XP_001648855; *Culex quinquefasciatus*, XP_001851442.

DjStag is expressed in *D. japonica* developing embryos

Northern blotting was conducted to assess the presence and size of the *DjStag* transcript. As shown in Figure 3, a transcript of approximately 1400 bp was detected, which proved that the gene we obtained was in the planarian *D. japonica*.

Freshwater planarians (Platyhelminthes: Tricladida) are divided into three families: Dugesiidae, Planariidae and Dendrocoelidae. Studies on the embryonic development of the family Dugesiidae were mainly pursued with *Schmidtea polychroa* (Bennazzi and Gremigni, 1982; Cardona et al., 2005, 2006). *D. japonica* belongs to the family Dugesiidae, whose members lay ectolecithal capsules. Several fertilized zygotes are deposited in egg capsules surrounded by large numbers of yolk cells. Cardona et al. (2005) defined an eight-stage system of embryonic development of triclads based on morphology. The embryonic development of *D. japonica* was similar with other triclad species. Thus, the eight stages were adopted in this paper.

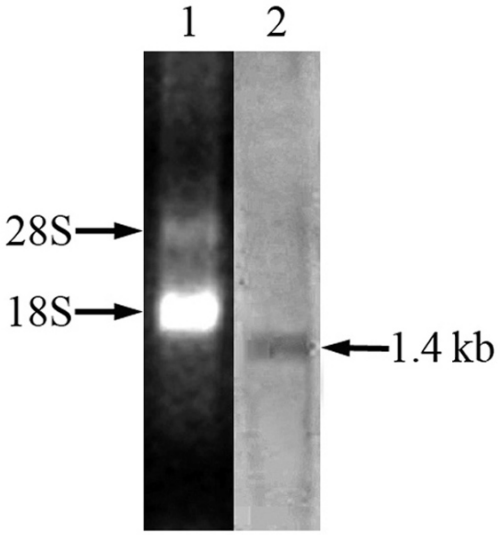

Figure 3. Northern blotting to detect *DjStag* RNA. *Lane 1* = total RNA of planarians. 28S and 18S rRNA bands are indicated. *Lane 2* = the blot was hybridized with DIG-labeled *DjStag* RNA probe. The arrow indicates the position of molecular size equivalent to 1.4 kb.

DjStag expression pattern in *D. japonica* during embryonic development was monitored by whole-mount *in situ* hybridization (Figure 4). The expression of *DjStag* mRNA was detected in the periphery of stage 3 embryos and in the embryonic pharynx (Figure 4a,b). At this stage, the primary epidermis has formed, covering the whole syncytium. The newly formed embryonic pharynx starts to ingest the external yolk cells into the syncytium. Due to this ingestion, a yolk-filled cavity is formed in the inner part of the embryo and the syncytium is restricted to the periphery of the embryo, which is called the germ band (Cardona et al., 2005; Solana et al., 2009).

During stage 4, embryos grow in size by continued yolk incorporation into the gut cavity (Cardona et al., 2005). *DjStag*-positive embryonic cells were found in the germ band (Figure 4c,d). In stage 5, embryonic cells start to differentiate into all definitive cell types. Due to the differentiation of muscle cells, previously spherical embryos flatten to a fat disk (Cardona et al., 2005; Solana and Romero, 2009). The number of *DjStag*-positive embryonic cells in the peripheral germ band increased (Figure 4e).

In stage 6, the syncytium has been disrupted and completely filled with embryonic differentiating cells. The embryos are more elongated in the antero-posterior (A-P) axis than in earlier stages (Cardona et al., 2005; Solana and Romero, 2009). In this stage, *DjStag* was mainly found in the dorsolateral region (Figure 4f,g). During stage 7, the embryo stretches, narrows further and becomes worm-shaped. The brain appears as two bilaterally symmetric hemispheres, and two pigmented eye cups form in the dorsal cortex of the brain (Cardona et al., 2005; Solana and Romero, 2009). *DjStag*-positive signals were detected in the dorsolateral regions along the A-P axis (Figure 4h,i,j).

In stage 8, the embryos show all the juvenile characteristics. The eyes, integument, musculature, nerve plexus, pharynx, and gut take on the characteristics they display in the

juvenile (Cardona et al., 2005). Expression was observed in the lateral region (Figure 4k), which will eventually become the parenchyma of the adult. At this stage, the embryo moves actively. It will spend a few days in the egg capsule if left undisturbed, but survives normally if dissected out (Cardona et al., 2005).

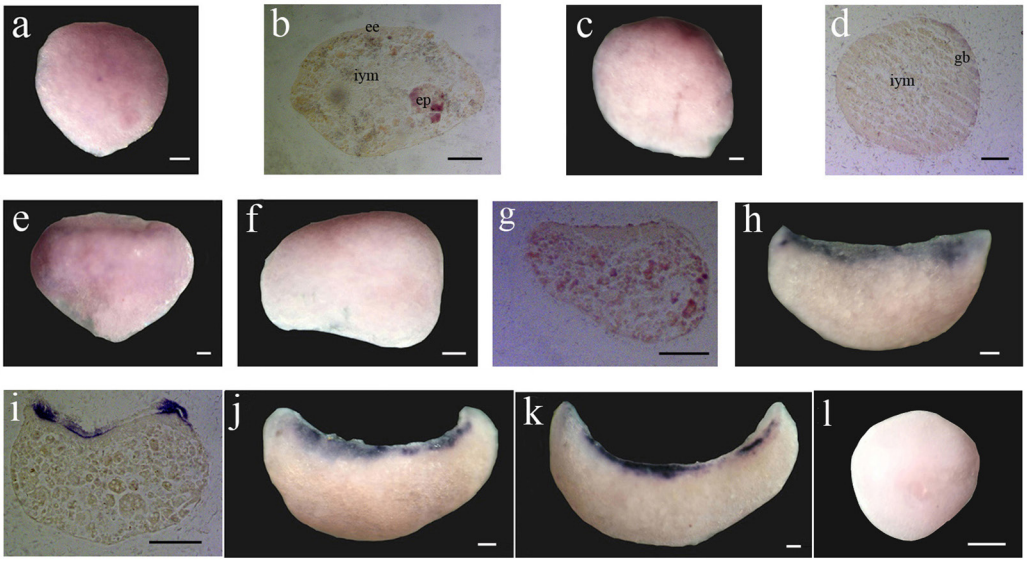

Figure 4. Expression patterns of *DjStag* mRNA in *Dugesia japonica* developing embryos detected by whole-mount *in situ* hybridization. **a.** Stage 3 embryo and **b.** section of a stage 3 embryo. The expression of *DjStag* mRNA is detected in embryonic pharynx and the periphery of embryos. **c.** Stage 4 embryo and **d.** section of a stage 4 embryo. *DjStag*-positive embryonic cells are found in the germ band. **e.** Stage 5 embryo. The number of *DjStag* -positive embryonic cells in the peripheral germ band increases. **f.** Stage 6 embryo and **g.** section of a stage 6 embryo. The *DjStag* is mainly found in the dorsolateral region. **h.** Early stage-7 embryo and **i.** section of a stage 7 embryo. *DjStag*-positive signals were detected in dorsolateral regions along the A-P axis. **j.** Stage 7 later embryo. **k.** Stage 8 embryo, expression is observed in the lateral region. **l.** Stage 3 embryo (control) processed and hybridized similarly with sense probes. No signal is seen in the control. In all images, anterior is to the left. ee = embryonic epidermis; gb = germ band; iym = inner yolk mass; ep = embryonic pharynx. Scale bars represent 100 μM.

DjStag is expressed in the juvenile planarians

Juveniles of *D. japonica* have very similar characteristics as the adult stage. The body wall encloses a thin parenchyma, the brain and nerve cords, the massive pharynx and metameric sets of protonephridia (Cardona et al., 2005). In the juvenile, *DjStag* mRNA was strongly expressed not only in the head and tail regions, but also in the parenchyma between the epidermis and the gastrodermis (Figure 5a,b,c), which will eventually become the parenchyma of the adult. In addition to this expression along the dorsal side, *DjStag* was expressed in discrete cells around the branches of the gastrovascular system (Figure 5a).

In triclad adults, much of the space between the gastrodermis and body wall is taken up by the parenchyma. Neoblasts are distributed throughout the planarian parenchyma. In addition, neoblasts are accumulated in multiple clusters along the midline and the lateral lines in

the dorsal parenchyma (Salvetti et al., 2000; Orii et al., 2005). During regeneration, neoblasts proliferate and accumulate beneath the wound epithelium, giving rise to the regenerative blastema (Salò and Baguñà, 2002; Rossi, et al., 2006). In addition to their role in regeneration, neoblasts are required for homeostasis in intact animals, where they serve to replace damaged or nonfunctional cells (Newmark and Sánchez Alvarado, 2002; Palakodeti et al., 2008).

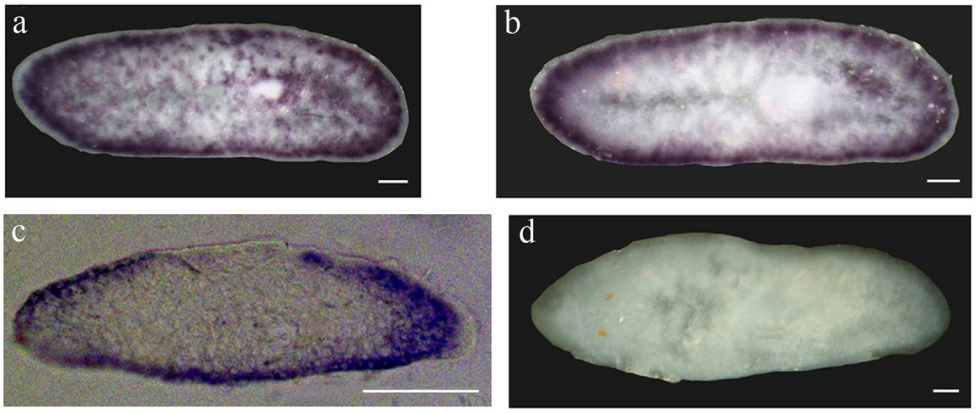

Figure 5. Expression patterns of *DjStag* mRNA in *Dugesia japonica* juveniles detected by whole-mount *in situ* hybridization. **a.** Juvenile in dorsal view and **b.** in ventral view; the *DjStag* mRNA was strongly expressed not only in the head and tail regions, but also in the parenchyma between the epidermis and the gastrodermis. **c.** Transversal section of a juvenile; the *DjStag* signals were expressed in the parenchyma. **d.** Dorsal view of a juvenile planarian (control) processed and hybridized similarly with sense probes. No signal is seen in the control juvenile. In all images, anterior is to the left. Scale bars represent 100 μM.

Previous study on STAG has been mainly focused on chromatid cohesion and segregation (Lara-Pezzi et al., 2004). In this study, *DjStag* was shown to be expressed from embryo to juvenile. *DjStag* was detected in the embryonic cells in the germ band at an early embryo stage. Later, it was mainly expressed in the parenchyma of the lateral region. In the juvenile, the most extensive expression of *DjStag* was observed in the discrete cells and the parenchyma between the epidermis and the gastrodermis. These results suggest that *DjStag* is expressed in the cellular subset that will become the neoblast cells of the adult worm. *DjStag* may play an essential role in cell division during planarian embryonic development.

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