

# Comprehensive gene expression analyses in pluripotent stem cells of a planarian, *Dugesia japonica*

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**ABSTRACT** The neoblasts are the only somatic stem cells in planarians possessing pluripotency, and can give rise to all types of cells, including germline cells. Recently, accumulated knowledge about the transcriptome and expression dynamics of various pluripotent somatic stem cells has provided important opportunities to understand not only fundamental mechanisms of pluripotency, but also stemness across species at the molecular level. The neoblasts can easily be eliminated by radiation. Also, by using fluorescence activated cell sorting (FACS), we can purify and collect many neoblasts, enabling identification of neoblast-related genes by comparison of the gene expression level among intact and X-ray-irradiated animals, and purified neoblasts. In order to find such genes, here we employed the high coverage expression profiling (HiCEP) method, which enables us to observe and compare genome-wide gene expression levels between different samples without advance sequence information, in the planarian *D. japonica* as a model organism of pluripotent stem cell research. We compared expression levels of ~17,000 peaks corresponding to independent genes among different samples, and obtained 102 peaks as candidates. Expression analysis of genes identified from those peaks by *in situ* hybridization revealed that at least 42 genes were expressed in the neoblasts and in neoblast-related cells that had a different distribution pattern in the body than neoblasts. Also, single-cell PCR analysis of those genes revealed heterogeneous expression of some genes in the neoblast population. Thus, using multidimensional gene expression analyses, we were able to obtain a valuable data set of neoblast-related genes and their expression patterns.

**KEY WORDS:** *planarian, pluripotent stem cell, HiCEP, comprehensive gene expression analysis*

## Introduction

Stem cells are extremely important cells not only during embryogenesis, but also during the life span of adult individuals. Fundamental features of stem cells are maintenance of the stem cell population and production of differentiated cells. These abilities are observed in the stem cells regardless of species and their differentiation ability. These general features imply the existence of common molecular mechanisms, underlying the regulation of stem cells among different tissues and across species in different taxa. In order to elucidate "stemness" in terms of the molecular signature of stem cells, comparative transcriptome analyses have

been conducted among various types of stem cells in mammals. Although it has been reported that adult somatic stem cells exist in many species of invertebrates as well as vertebrates (Agata *et al.*, 2006), comparative gene expression analysis across those species, especially in invertebrates, is still poor because of lack of information about stem cell-specific gene sets in various invertebrates. Examining common features of stemness across species at the molecular level, however, might bring us very important knowledge not only about stem cell biology, but also regarding

*Abbreviations used in this paper:* FACS, fluorescent activated cell sorting; FBSC-PCR, FACS-based single cell PCR; HiCEP, high coverage expression profiling.

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evo-devo or medical applications.

*Dugesia japonica*, a freshwater planarian species, has robust regenerative ability, by which almost any small fragments from the body except for in front of the eyes and in the pharynx can regenerate into complete functional animals within 1 week after amputation. Adult stem cells in freshwater planarians, called as neoblasts, can give rise to all types of somatic cells during regeneration (Newmark & Sánchez Alvarado 2000). Also, germline cells are produced from the neoblasts in the adult body (Sato et al., 2006). Furthermore, the neoblasts supply differentiated cells in intact animals during tissue homeostasis (Newmark and Sánchez Alvarado 2000). X- or gamma-ray irradiation can specifically eliminate the neoblasts, and irradiated animals lose their regenerative ability. Transplantation of a neoblast-rich fraction can restore the regenerative ability of the X-ray-irradiated animals, indicating that regeneration of planarians depends on the neoblasts (Baguñà et al., 1989). In addition to those intriguing stem cell features in planarians, recent methodological advances such as *in situ* hybridization for mRNA detection, lineage tracing by BrdU-labeling, RNA interference (RNAi) for gene knockdown, purification of the neoblasts by fluorescence activated cell sorting (FACS) and single cell PCR based on FACS, make planarians a good animal for stem cell research (Hayashi et al., 2006; Hayashi et al., 2010; Newmark and Sánchez Alvarado 2000; Sánchez Alvarado and Newmark 1999; Umesonon et al., 1997).

Thus, much research at the molecular level on the neoblasts has been reported, and information about the expression and function of specific genes is accumulating. The foremost molecular feature of the neoblasts in *D. japonica* is the expression and functions of a number of RNA-binding proteins encoded by germline-specific genes (Shibata et al., 2010). Vasa, PL10/DDX3 and Me31B are DEAD box RNA helicases expressed in germline cells in other animals. In *D. japonica*, those genes, *Djvas-1*, *DjvlgA* and *Djcbc-1* respectively, are expressed in the neoblasts (Rouhana et al., 2010; Shibata et al., 1999; Yoshida-Kashikawa et al., 2007). Also, *Djpum*, a member of the PUF-domain family that acts in germline cells in flies and nematodes, is expressed and functions in the neoblasts (Salveti et al., 2005). Furthermore, expression of *piwi* homolog genes, a subfamily of the Argonaute family whose mutants show severe defects of the germline in flies and mice, and expression of *bruli*, encoding a member of the Bruno-like family of proteins that represses translation of *osk* in germline cells in flies, is observed in the neoblasts in *D. japonica* as well as in *Schmidtea mediterranea*, a related planarian species (reviewed by Shibata et al., 2010). RNAi experiments revealed that RNA-binding proteins, including those encoded by germline-specific genes, are involved in the maintenance, proliferation or differentiation of the neoblasts in planarians (reviewed by Shibata et al., 2010).

Also, *minichromosome maintenance2, 3* (*DjMCM2, 3*) and *proliferating cell nuclear antigen* (*Djpcna*) related to the process of DNA replication during cell proliferation have been reported to be neoblast-specific genes in *D. japonica* (Oriei et al., 2005; Salvetti et al., 2000). All of these gene identifications are based on homology with known genes in other animals. Although a considerable number of neoblast-specific genes have been obtained by such homology-based methods, such approaches have some biases depending on each researcher that might make it difficult to obtain the overall gene expression profile of the neoblasts. To resolve this issue, genome-wide comprehensive gene expression analysis would be a beneficial approach.

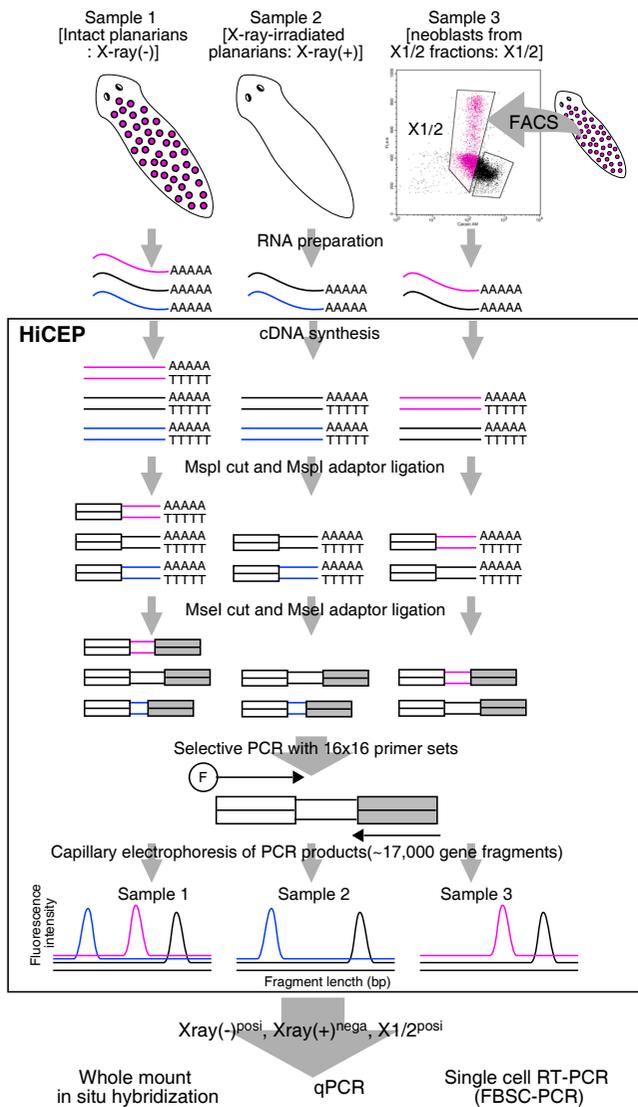
The major method of comprehensive gene expression analysis comparing expression levels between different samples is hybridization-based assays such as microarray analysis. Although microarrays can compare the expression of numerous genes between different samples, the method needs sequence information of a gene set from an EST or genome database. Indeed, microarray experiments using an EST database in *D. japonica* (using a set of 600 genes) and *S. mediterranea* (using a set of 3,435 genes) could identify many genes expressed in the neoblasts by comparison of the gene expression level between intact and X-ray irradiated planarians (Eisenhoffer et al., 2008; Rossi et al., 2007). Also, a method of transcriptome analysis that does not require any advance sequence information about gene set is available. Because this method, named HiCEP (high coverage expression profiling) has been developed through substantial improvement of the amplified fragment length polymorphism (AFLP), we can detect individual genes as different lengths of PCR fragments by electrophoresis genome widely, and can compare the expression levels of each gene between samples as a difference of fluorescent intensity without any sequence information (Fukumura et al., 2003). In embryonic stem cells (ES cells), about 40,000 transcripts including novel and non-coding genes have been identified by HiCEP (Araki et al., 2006). Thus, HiCEP is a suitable method for accumulating information about gene expression in organisms that have incomplete or lack of gene sequence information.

Here, we report genome-wide comprehensive comparison of gene expression between intact planarians, X-ray-irradiated planarians and purified neoblasts using FACS by HiCEP. Also, we confirmed the expression of HiCEP clones by several methods: *in situ* hybridization, quantitative RT-PCR (qPCR) and FACS-based single cell PCR (FBSC-PCR). The results showed that we could obtain many neoblast specific genes which showed different expression patterns, and also could demonstrate heterogeneity of the neoblasts.

## Results

### Preparation of samples and experimental procedure for HiCEP analysis in *D. japonica*

In order to identify neoblast-specific genes, we used three samples, namely, intact and X-ray-irradiated planarians and purified neoblasts. Planarians were irradiated with 160 cGy of X-rays, which was a sufficient dose to eliminate the neoblasts completely (Yoshida-Kashikawa et al., 2007). X-ray-irradiated animals lost regenerative ability, and then died within 3 weeks after irradiation. Cells of *D. japonica* have been classified into three cell fractions, X1, X2 and XIS, according to their size and DNA contents by FACS (Hayashi et al., 2006). The X1 fraction is composed of neoblasts in S/G2/M-phase of the cell cycle (Hayashi et al., 2006, 2010). X2 is a mixed fraction of the neoblasts in G1-phase and some differentiated cells, and the XIS fraction comprises several types of differentiated cells (Hayashi et al., 2006, 2010). We collected about  $1 \times 10^7$  cells from the mixed-fraction of X1 and X2 for HiCEP analysis as a neoblast-rich sample. First, we prepared total RNA from non-irradiated [X-ray(-)], X-ray irradiated animals [X-ray(+)] and cells from the X1 and 2 fractions (X1/2, Fig. 1). Synthesized cDNAs of each sample were digested with MspI, followed by MspI adaptor ligation (Fig. 1). The cDNAs from poly (A) + RNAs were collected and digested with MseI, followed by ligation of MseI adaptor



**Fig. 1. Schematic drawing of the high coverage expression profiling (HiCEP) experimental procedure.** For details see text.

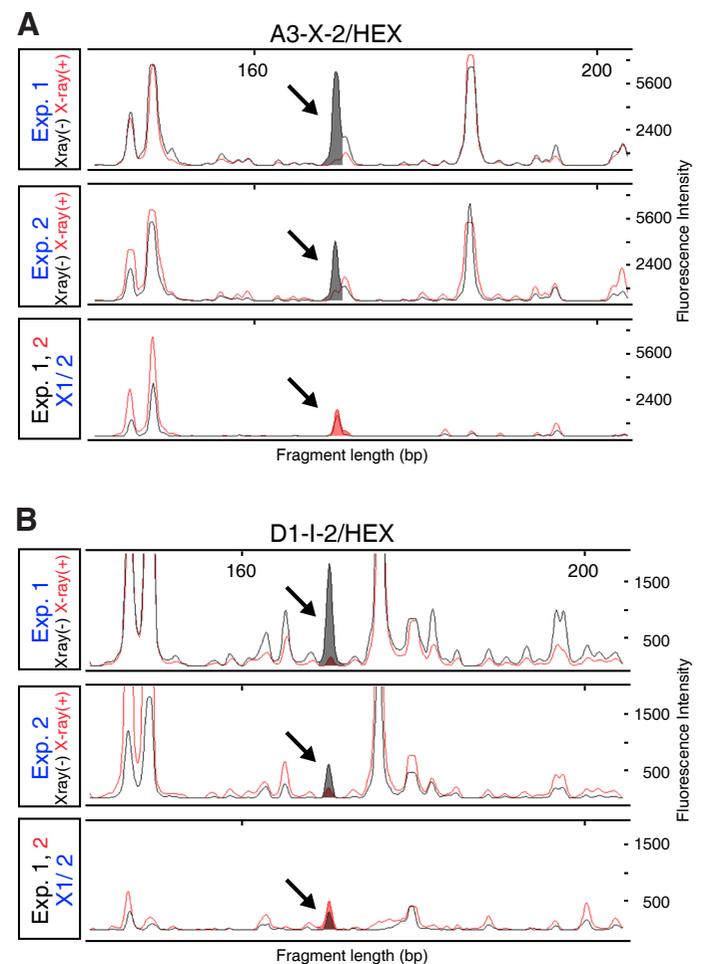
(Fig. 1). Selective PCR using the 16x16 primer sets was carried out, and the products were analyzed by capillary electrophoresis (Fig. 1: Fukumura *et al.*, 2003). As a result, we detected each PCR product as a peak with a particular length and intensity in each sample. We expected that the peaks of candidate genes would be positive in the non-irradiated planarians, negative or decreased in the X-ray-irradiated planarians, and positive in the neoblast-rich sample [ $X\text{-ray}(-)^{\text{posi}}$ / $X\text{-ray}(+)^{\text{nega}}$ / $X1/2^{\text{posi}}$ ], as indicated for the red peak in Fig. 1. The obtained candidate genes were then subjected to the following expression analyses (Fig. 1).

#### Comparison of gene expression levels among the different samples by HiCEP in *D. japonica*

We performed HiCEP analysis twice for each sample to check the reproducibility. Consequently, we detected about 17,000 peaks in total in the X-ray(-) sample. The expression levels of 318 peaks showed an  $X\text{-ray}(-)^{\text{posi}}$ / $X\text{-ray}(+)^{\text{nega}}$  pattern [more than 50% reduction in the X-ray(+)]. Among them, 102 peaks showed the expected pattern:  $X\text{-ray}(-)^{\text{posi}}$ / $X\text{-ray}(+)^{\text{nega}}$ / $X1/2^{\text{posi}}$  (table S1). For example,

the peak named A3-X-2/HEX was greatly reduced in the X-ray(+) sample in the two experiments (Fig. 2A). A peak with same length was observed in the X1/2 sample (Fig. 2A). Also, the peak D1-I-2/HEX showed a similar result (Fig. 2B).

By sequence analysis of several peaks, 110 kinds of gene fragments from the 102 peaks were found, indicating that some peaks were composed of multiple fragments, as was also observed in the transcriptome analysis of ES cells by HiCEP (Araki *et al.*, 2006; Table S1). Among them, 86 fragments were matched to clones in the EST database of *D. japonica*. We found that at least 13 fragments were derived from 6 kinds of EST clones, suggesting that there was polymorphism or alternative splicing in those clones. Fifty of the 78 independent EST clones matched HiCEP fragments showing homology or similarity to genes in other animals (Table S1). Although we extended some fragment sequences, which did not match to any EST clones, by 5'RACE, such as #002 (*C3-I-2/NED*) and #005 (*G4-I-2/HEX*), we did not find clone matching those fragments in the EST database or any homology with genes



**Fig. 2. Examples of comparison of expression level of gene fragments between X-ray(-), X-ray(+), and X1/2 samples by HiCEP.** (A) Expression pattern of A3-X-2/HEX. (B) Expression pattern of D1-I-2/HEX. Black peaks in experiments 1 and 2 (Exp. 1 and 2) indicate expression levels of genes in the X-ray(-) sample. Red peaks in experiments 1 and 2 (Exp. 1 and 2) indicate expression levels of genes in the X-ray(+) sample. Black and red peaks in Exp. 1, 2 indicate gene expression in the X1/2 sample. Arrows indicate gene fragments showing the  $X\text{-ray}(-)^{\text{posi}}$ / $X\text{-ray}(+)^{\text{nega}}$ / $X1/2^{\text{posi}}$  pattern.

in other animals (Table S1). For the other non-matched fragments, 5'RACE failed to extend the sequence. Then, the EST-matched clones and the 2 non-matched clones were subjected to the next analysis.

#### Expression analyses of HiCEP genes by *in situ* hybridization

To confirm the stem-cell-expressed genes identified by HiCEP analysis, called HiCEP clones, we performed whole-mount *in situ* hybridization (Fig. 3). The results showed that 42 independent HiCEP clones derived from 46 HiCEP fragments were specifically expressed in X-ray-sensitive cells. Among those clones, 16 did not show any homology or similarity with any known genes in

other organisms (Table 1), whereas #001 (unknown) was similar to NB.32.1g, a novel gene in *S. mediterranea* whose function is not clear. (Eisenhoffer *et al.*, 2008). As mentioned above, many kinds of RNA-binding protein genes, including germline-specific genes, are expressed in the neoblasts of *D. japonica* (reviewed by Shibata *et al.*, 2010). In our analyses, #008 (*piwiA*; a homolog of *piwi*, Yoshida-Kashikawa *et al.*, 2007, Hayashi *et al.*, 2010, Rouhana *et al.*, 2010) and #028 (a DEAD box family gene) were obtained as genes containing RNA-binding motifs (Table S1). Also, we found a histone RNA hairpin-binding protein [#024(*hRNAhbp*)], which is required for translation of histone mRNA (Allard *et al.*, 2005), and two ribosomal proteins [#013 (*rpL44*; a ribosomal pro-

TABLE 1

## LIST OF THE HiCEP CLONES EXPRESSED IN THE NEOBLASTS

Fragment ID	Fragment length (bp)	Clone #	Homology (species)	Published Clone ID in EST (gene name)	E value
A3-X-2/HEX	169.62	001	NB.31.1g [Schmidtea mediterranea]	05955HH	4E-16
C3-I-2/NED	104.64	002	N/A		
D3-I-2/HEX	169.73	003	NB.31.1g [Schmidtea mediterranea]	=#001	
E11-I-2/FAM	106.61	004	N/A	05656HH	
G4-I-2/HEX	226.98	005	N/A		
E5-I-2/HEX	163.38	006	zinc finger protein Ci-ZF(C2H2)-42 [Ciona intestinalis]	02488HH	7E-11
C7-I-2/NED	262.55	007	SWI/SNF-related chromatin binding protein [Schistosoma mansoni]	02960HH	4E-29
E6-I-2/HEX	107.23	008	Piwi [Botryllus primigenus]	03609HH (DjpiwiA)	2E-59
E5-I-2/HEX	229.27	009	arginine N-methyltransferase 1 [Echinococcus granulosus]	04361HH	2E-57
D3-I-2/FAM	318.17	010	Nucleoside diphosphate kinase [Aspergillus nidulans FGSC A4]	06317HH	4E-51
H6-I-2/FAM	236.91	011	purinergic receptor P2X1 [Taeniopygia guttata]	06727HH	3E-21
B3-I-2/FAM	177.92	012	purinergic receptor P2X4 [Sus scrofa]	06974HH (DjP2X-A)	9E-25
H8-I-2/NED	196.11	013	ribosomal protein L44 [Chlamys farreni]		1E-41
A12-X2-1/NED	170.48	016	N/A		
D12-I-2/NED	161.72	019	Zn finger homeodomain 2 [Tribolium castaneum]		1E-12
B1-I-2/FAM	198.82	023	selenophosphate synthetase 1 [Mus musculus]		4E-43
B7-I-2/HEX	160.42	024	Histone RNA hairpin-binding protein [Schistosoma japonicum]		2E-29
F2-I-2/FAM	200.92	025	N/A		
F10-I-2/NED	138.39	028	similar to DEAD (Asp-Glu-Ala-Asp) box polypeptide 53 [Ciona intestinalis]		0.000003
F12-I-2/NED	138.4	029	similar to DEAD (Asp-Glu-Ala-Asp) box polypeptide 53 [Ciona intestinalis]	=#028	
F1-I-2/FAM	430.13	030	Rootletin (Ciliary rootlet coiled-coil protein) [Schistosoma mansoni]		1E-144
G6-I-2/HEX	212.56	032	topoisomerase (DNA) II beta [Mus musculus]		3E-32
H4-I-2/FAM	160	033	N/A		
H6-I-2/FAM	215.14	035	Sox8b [Takifugu rubripes]		1E-20
D9-I-2/NED	469.49	037	Placenta-specific gene 8 protein [Schistosoma japonicum]		1E-13
E9-I-2/FAM	244.37	038	SMED P53 [Schmidtea mediterranea]		
A11-X2-2/FAM	105.56	040	Setb protein [Danio rerio]	02457HH	8E-49
A4-X2-1/HEX	276.93	041	similar to DEAD (Asp-Glu-Ala-Asp) box polypeptide 53 [Ciona intestinalis]	=#028	
B5-I-2/NED	123.59	042	Putative OPA3-like protein CG13603 [Schistosoma japonicum]		4E-32
D3-I-2/FAM	179.4	046	Dynein intermediate chain 2 [Anthocidarid crassispina]	03394HH	5E-57
D9-I-2/FAM	101.36	047-2	chaperonin containing t-complex protein 1 gamma subunit tpcg [Schistosoma mansoni]	03940HH	0.0
E5-I-2/HEX	99.47	050	large subunit ribosomal protein 10 [Oigolaimella attenuata]	04458HH	8E-59
E6-I-2/FAM	147.06	051	N/A	00998HH	0.97
F10-I-2/NED	76.56	054	N/A		
H3-I-2/NED	60.99	057	N/A		
H6-I-2/FAM	62.08	058	N/A	06441HH	
A6-I-2/NED	89.7	060	N/A		
C3-I-2/FAM	84.12	061-2	NADH dehydrogenase subunit 2 [Metaseiulus occidentalis]	07117HH	0.005
C7-I-2/NED	55.94	062	peroxiredoxins, prx-1, prx-2, prx-3 [Aedes aegypti]		1E-81
E10-I-2/FAM	67.99	075	N/A	00310HH	
G11-I-2/FAM	106.67	081	N/A	=#004	
G2-I-2/FAM	64.87	082	protein phosphatase 2C [Schistosoma mansoni]	00503HH	3E-62
G6-I-2/FAM	99.35	085	conserved-hypothetical-protein-[xodes-scapularis]		0.000006
H1-I-2/FAM	71.52	097	similar-to-cytochrome-P450-CYP15A1-[Acyrtosiphon-pisum]		0.00000004
H5-I-2/FAM	54.49	098	CDGSH-type Zn finger-containing protein-like protein [Schistosoma mansoni]		1E-31
G12-I-2/FAM	91.73	100	FK506-binding protein [Manduca sexta]		2E-42

tein L44 homolog), #050 (*rp10*; a large subunit ribosomal protein 10 homolog). Notably, #007 and #009 clones showed similarity with epigenetics-related proteins, SWI/SNF-related chromatin binding protein (*SWI/SNF*) and arginine N-methyltransferase (*PRMT*), respectively (Table 1). Furthermore, transcription factors and zinc finger proteins were found among the clones [#006 (*zf*, a planarian zinc finger protein), #035 (*soxP1*, a planarian homolog of *sox* gene), #038 (*p53*, a homolog of *p53*) #098 (*zf*, a planarian zinc finger protein)]. Also, other kinds of genes were obtained by HiCEP as genes expressed in X-ray-sensitive cells (Table 1), for example, a membrane protein P2X homolog, #012 (*P2X-A*) or protein phosphatase 2C, #082 (*pp2C*), indicating that a lack of bias in the kind of gene obtained by HiCEP.

#008 (*piwiA*), showed a typical pattern of neoblast-specific expression, in which the distribution of positive cells was observed in the mesenchymal space from behind the eyes to the tail (Fig. 3C; Yoshida-Kashikawa *et al.*, 2007; Rouhana *et al.*, 2010; Shibata *et al.*, 2010). Cells expressing #008 (*piwiA*) completely disappeared in the X-ray-irradiated animals (Fig. 3D), indicating that this gene was specifically expressed in the neoblasts, as previously described (Yoshida-Kashikawa *et al.*, 2007; Rouhana *et al.*, 2010; Shibata *et al.*, 2010). Genes such as #012 (*P2X-A*) and #032 (unknown) showed the same expression patterns as #008 (*piwiA*) in intact and X-ray-irradiated animals (data not shown). A similar expression pattern to that of #008 (*piwiA*) was observed for #009 (*PRMT*) (Fig. 3E). Although the expression pattern of #009 (*PRMT*) appeared to be identical to that of the neoblast-specific genes in intact animals, in X-ray-irradiated animals, expression in brain neurons was still observed (Fig. 3F). Many HiCEP clones showed this expression pattern, for example, #005 (unknown), #007 (*SWI/SNF*), #057 (unknown), and #100 (*FK506-binding protein*) (data not shown).

Although expression of #001 (unknown) disappeared in X-ray-irradiated animals (Fig. 3B), the expression pattern of this clone was different from that of #008 (*piwiA*) or #009 (*PRMT*) (Fig. 3A). Cells expressing #001 (unknown) were located in the mesenchymal space from in front of the eyes to the tail. The neoblasts were lacking in front of the eyes (Fig. 3C), suggesting that the cells-expressing #001 (unknown) are another type of X-ray-sensitive cells. Also, #038 (*p53*) showed a similar expression pattern to that of #001 (unknown).

We found ubiquitous expression patterns of some HiCEP clones, for example #015 (*rpS3*; a homolog of *ribosomal protein S3* in *D.japonica*) in intact animals (Fig. 3G). Ubiquitous expression of #015 (*rpS3*) was also observed in X-ray-irradiated animals, although the expression level of #015 (*rpS3*) seemed to be decreased (Fig. 3H). According to these expression patterns, we mainly classified HiCEP clones into 3 types: Type 1, represented by the expression pattern of #001 (unknown); Type 2, represented by the #008 (*piwiA*) or #009 (*PRMT*) expression patterns, and Type 3, represented by the expression pattern of #015 (*rpS3*). Type 2 could be further categorized into 2 more types according to whether the gene was expressed in the brain or not. Then we named the genes that showed neoblast-specific expression Type 2A, and that genes that were expressed in both neoblasts and brain Type 2B. Thus, 2 and 40 kinds of genes were categorized into Type 1 and Type 2, respectively.

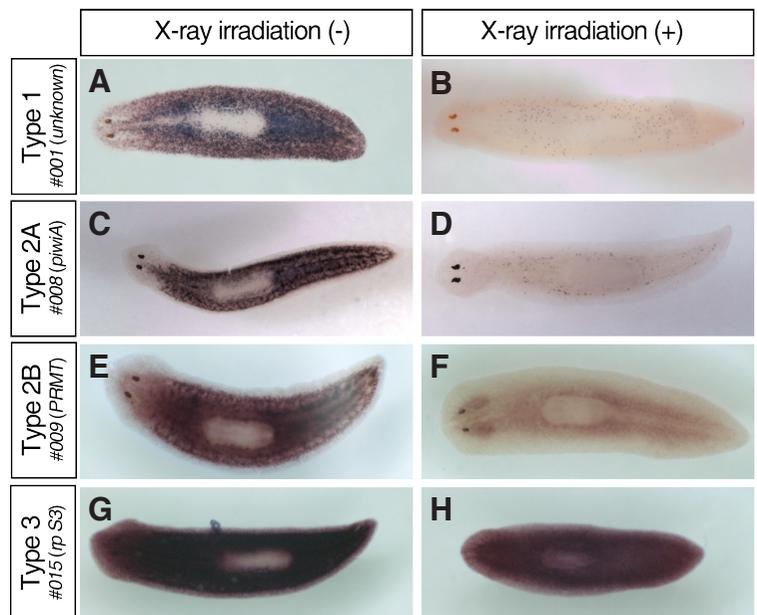
### Confirmation of expression level of several types of genes by qPCR

The expression of various HiCEP genes was also confirmed by qPCR (Fig. 4). The expression levels of Type 1 and Type 2A HiCEP clones were significantly decreased in the X-ray-irradiated planarians (Fig. 4). Also, the gene expression levels of Type 2B HiCEP clones were greatly decreased, like those of Type 1 and Type 2A clones; however, about 20 % of the expression of some clones, for example #009 (*PRMT*), remained in the X-ray-irradiated animals, where it was mainly seen expression in the brain (Fig. 4). Also, about a 40% reduction of the gene expression level of a Type 3 clone, #015 (*rpS3*), was observed in the X-ray-irradiated planarians. In contrast with these changes of the gene expression levels of HiCEP clones, the expression levels of differentiated cell marker genes, e. g., *Djsyt* as a neural marker and *DjMHC-A* as a muscle marker were not affected by X-ray irradiation (Fig. 4).

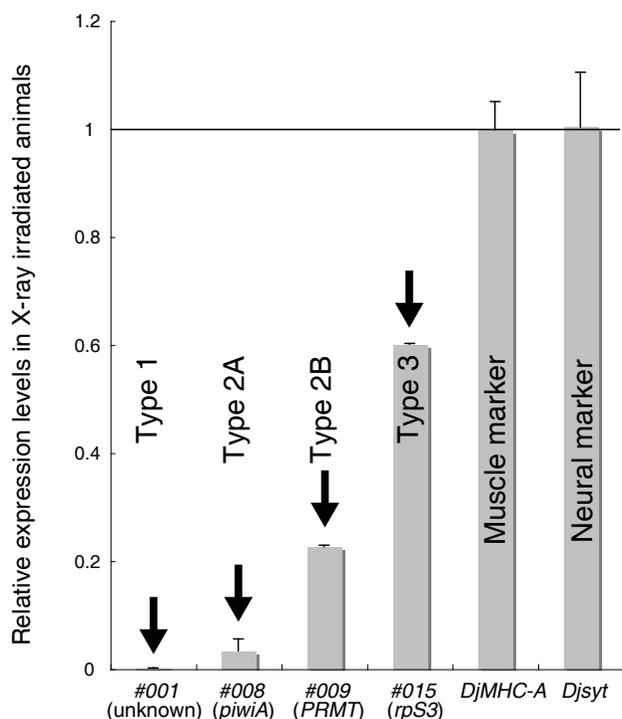
### Expression analysis of HiCEP clones by single cell RT-PCR

Finally, we employed FACS-based single cell RT-PCR (FBSC-PCR) to investigate the expression of the neoblast-specific HiCEP clones at the single cell level. FBSC-PCR enables us to analyze the expression of many genes in single cells, and to link the gene expression information to the FACS profile using the index-sorting function (Hayashi *et al.*, 2010). We collected 188 cells each from the X1, X2 and XIS fractions (Fig. 5A), and analyzed the gene expression of several HiCEP clones in these cells.

#008 (*piwiA*) was expressed in 84% of the cells in the X1 fraction and in 34% of the cells in the X2 fraction consistent with the previous result (Fig. 5B; Hayashi *et al.*, 2010). Cells expressing



**Fig. 3. Expression of Type 1, 2 and 3 genes revealed by whole mount *in situ* hybridization.** (A) Expression pattern of Type 1 gene [#001 (unknown)] in intact animal. (B) Expression pattern of #001 (unknown) in X-ray-irradiated planarian. (C) Expression pattern of Type 2A gene [#008 (*piwiA*)] in intact animal. (D) Expression pattern of #008 (*piwiA*) in X-ray-irradiated planarian. (E) Expression pattern of Type 2B gene [#009 (*PRMT*)] in intact animal. (F) Expression pattern of #009 (*PRMT*) in X-ray-irradiated planarian. (G) Expression pattern of Type 3 gene [#015 (*rpS3*)] in intact animal. (H) Expression pattern of #015 (*rpS3*) in X-ray-irradiated planarian.



**Fig. 4. Confirmation of expression levels of several types of genes by qPCR.** 1 on the Y axis indicates relative expression level in intact planarians.

#008 (*piwiA*) were specifically sorted into the X1 and X2 fractions, in accord with the results from *in situ* hybridization (Fig. 5B). Another Type 2A gene, #012 (*P2X-A*), was also specifically expressed in cells in the X1 and X2 fractions, and almost all #012 (*P2X-A*)-expressing cells co-expressed #008 (*piwiA*); however, the number of #012 (*P2X-A*)-expressing cells was about half of the number of #008 (*piwiA*)-expressing cells (Fig. 5 C,H). 50.6% (80/158) of #008 (*piwiA*)-expressing cells in the X1 fraction co-expressed #012 (*P2X-A*), and 27.7% (18/65) of #008 (*piwiA*)-expressing cells in the X2 fraction were also positive for #012 (*P2X-A*), indicating heterogeneity of the neoblasts. Expression of #007, a Type 2B gene, was also detected in cells in the X1 and X2 fractions (Fig. 5D). Almost all cells expressing #008 (*piwiA*) in the X1 and X2 fractions expressed #007 (*SWI/SNF $\alpha$* ) [94.9% (150/158) and 75.4% (49/65), respectively] (Fig. 5G). Also, we found that 34 cells in the X2 fraction were #007 (*SWI/SNF $\alpha$* )-single positive cells as well as 36 cells in the X1S fraction (Fig. 5 D,G). Those #007 (*SWI/SNF $\alpha$* )-single positive cells are expected to be neuronal cells according to the results of *in situ* hybridization. Indeed, 55.6% of #007 (*SWI/SNF $\alpha$* )-single positive cells in the X1S fraction co-expressed neuronal marker genes, whereas some #007 (*SWI/SNF $\alpha$* )-single positive cells in the X2 fraction were positive for not only neuronal markers, but also muscular or mucus-producing cell marker genes, suggesting that #007 (*SWI/SNF $\alpha$* )-single positive cells in the X2 fraction might be differentiating cells from the neoblasts (data not shown). A similar expression pattern and sorting profile were observed in the case of #009, another Type 2B clone, in which 91.2% of cells in the X1 fraction and 69.2% of cells in the X2 fraction co-expressed both *DjpiwiA* and #009 (*PRMT*) (Fig. 5E). Also, about 30% of #009-single positive cells in the X1S fraction were positive for neuronal cell marker genes (data not shown). We also

found heterogeneous gene expression of a Type 2B clone, #004 (unknown), which showed no homology with any known gene. 35.4% (56/158) and 29.2% (19/65) of cells expressing #008 (*piwiA*) in the X1 fraction and X2 fraction, respectively, co-expressed #004 (unknown) (Fig. 5F). Furthermore, expression of #012 (*P2X-A*) was detected in 55.4% of cells co-expressing *DjpiwiA* and #004 (unknown) in the X1 fraction (Fig. 5H), showing the complexity of the neoblast population.

## Discussion

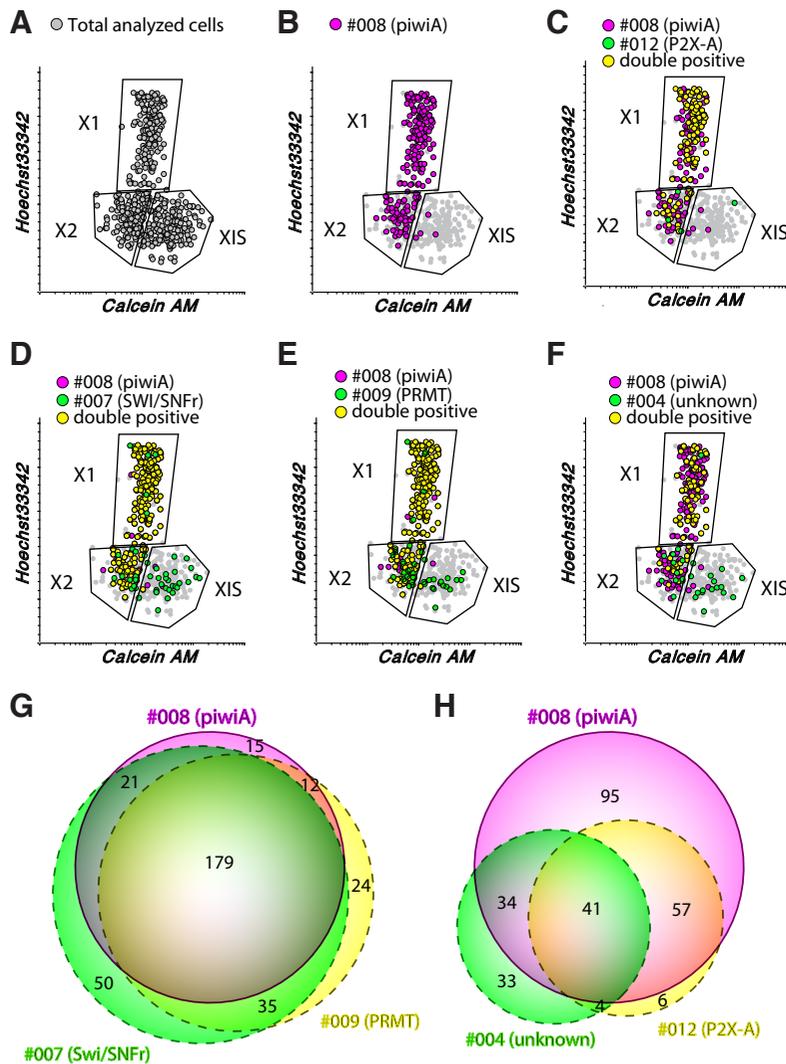
Genome-wide approaches by transcriptome analysis yield significant biological information for understanding molecular fundamentals underlying complex biological phenomena. Also, accumulation of transcriptomic information in several species belonging to different phyla gives us a great opportunity to shed light on the commonality or differences of molecular machinery regulating the same cell type or same phenomenon between species. In order to accumulate transcriptomic information of the neoblast in *D. japonica*, we conducted genome-wide gene expression analysis by HiCEP, which can survey gene expression genome widely and detect the expression levels of those genes simultaneously (Fukumura et al., 2003). Using this unlimited gene expression analysis, we detected ~17,000 peaks in the intact sample. So far, the genome size of *D. japonica* is estimated to be about 1~1.5 G bp, and the total number of genes is expected to be around 20,000 (Agata and Nishimura, personal communication). Actually, it was observed that some different peaks were derived from the same gene in our results, indicating that the number of peaks did not reflect the number of kinds of genes (Table 1 and S1). However, most of the peaks matched to independent EST clones, suggesting that our analysis could survey genome widely.

### Comparison of gene expression levels in the three samples by HiCEP

By comparison of the gene expression in 5 distinct samples by HiCEP, namely from head-regenerating tissues 12 and 24 hours after amputation, tail-regenerating tissues 12 and 24 hours after amputation, and non-regenerating tissues, Tasaki et al., succeeded in the identification of *DjmkpA* (*Dugesia japonica* mitogen-activated kinase phosphatase A) as an early blastema-specific gene (Tasaki et al., 2011). Also in this report, we compared gene expression levels between 3 distinct samples [X-ray(-), X-ray(+) and X1/2], and detected 102 peaks as candidates which showed the expression pattern X-ray(-)<sup>posi</sup>/X-ray(+)<sup>nega</sup>/X1/2<sup>posi</sup>, among the 318 peaks of X-ray(-)<sup>posi</sup>/X-ray(+)<sup>nega</sup>. The remaining 216 peaks showed an X-ray(-)<sup>posi</sup>/X-ray(+)<sup>nega</sup>/X1/2<sup>nega</sup> pattern (data not shown). *In situ* hybridization revealed that a clone of this type was not expressed in the neoblasts (data not shown). Thus, we could narrow down the number of candidate peaks by comparing the gene expression in not only intact and X-ray-irradiated planarians, but also the stem cell-enriched sample collected by FACS.

### Genes obtained by HiCEP

HiCEP enables us to obtain not only known genes, but also novel genes (Araki et al., 2006). In our analysis, we could not find any EST clone matching 24 fragments (Table S1). The sequence of these fragments also did not show any similarity with any known gene in other organisms, although at least 2 clones extended



**Fig. 5. Single cell gene expression analyses by FACS-based single cell PCR (FBSC-PCR).** (A) Total cells in the several fractions used for analysis. (B) Expression of #008 (piwiA). (C) Co-expression analysis of #012 (P2X-A) (green) and #008 (piwiA) (magenta). Yellow dots show double positive cells. (D) Co-expression analysis of #007 (SWI/SNFr) (green) and #008 (piwiA) (magenta). Yellow dots show double positive cells. (E) Co-expression analysis of #009 (PRMT) (green) and #008 (piwiA) (magenta). Yellow dots show double positive cells. (F) Co-expression analysis of #004 (unknown) (green) and #008 (piwiA) (magenta). Yellow dots show double positive cells. (G) Co-expression diagram for #008 (piwiA) (magenta), #007 (SWI/SNFr) (green) and #009 (PRMT) (yellow). The numbers indicate the cell number belonging to each overlapping expression. (H) Co-expression diagram for #008 (piwiA) (magenta), #004 (unknown) (green) and #012 (P2X-A) (yellow). The numbers indicate the cell number belonging to each overlapping expression.

from the fragments by 5'RACE among them were expressed in the neoblasts [#002 (unknown) and #005 (unknown)]. Genome information and enrichment of the EST database in *D. japonica* might uncover gene information about the remaining fragments in the future. Thus, we could identify many genes expressed in the neoblasts, not only known genes (30 independent clones), but also novel genes (12 independent clones) by HiCEP analysis.

Previously, microarray analysis (using the Dj600 chip) to obtain neoblast-specific genes in *D. japonica* was carried out (Rossi *et al.*, 2007). In the results, 42 kinds of genes, including 4 genes

known to be expressed in the neoblasts, showed reduced expression levels in X-ray-irradiated planarians. Although our analyses revealed that 42 kinds of genes were expressed in the neoblasts or related cells, only 2 genes were overlapping the results from the Dj600 chip and HiCEP [#010 (*ndpk*; nucleoside diphosphate kinase) and #040 (*setb*)]. The foremost difference in the results between the Dj600 chip and HiCEP is that unknown (novel) genes expressed in the neoblasts can be found in the result of HiCEP, because the HiCEP method is independent of sequence information.

#### In situ hybridization revealed different expression patterns among HiCEP clones

We found different expression patterns among HiCEP clones via expression analysis by *in situ* hybridization. We categorized those expression patterns mainly into 3 types: Type 1, Type 2 and Type 3 (Fig. 3). Although type 2 was the typical distribution pattern of the neoblasts, this pattern was subdivided into 2 more types: Type 2A and B, according to difference of expression in the brain. Expression pattern type 2B, which is expression in the neoblasts and brain neurons, was also observed in the results of extensive analyses of RNA-binding proteins, as well as Type 2A (Rouhana *et al.*, 2010; Yoshida-Kashikawa *et al.*, 2007), suggesting that there are similar molecular machineries to regulate the neoblasts and neurons (Yoshida-Kashikawa *et al.*, 2007).

Type 3 clones were ubiquitously expressed in planarians, but their expression levels were reduced by X-ray irradiation. #015 (*rpS3*) showed a Type 3 expression pattern. rpS3 is known to be a component of the small ribosomal subunit involved in translational initiation (Polakiewicz *et al.*, 1995). Interestingly, multiple functions of rpS3 in addition to translation have been reported, for example, rpS3 is involved in DNA repair after UV irradiation (Jung *et al.*, 2001; Lee *et al.*, 2002). Also, rpS3 binds to p53 and MDM2, and regulates the p53 level by preventing its degradation (Yadavilli *et al.*, 2009), suggesting that rpS3 is also concerned with apoptosis and cell cycle regulation via the p53 cascade. The neoblasts are a unique somatic cell population possessing proliferative ability, and show sensitivity to X-ray or gamma ray irradiation that is expected to induce DNA breaks and apoptosis (Pellettieri *et al.*, 2009). This implies that #015 (*rpS3*) expression in the neoblasts might be relatively higher than that in differentiated cells. This may cause a decrease of the expression level in the X-ray-irradiated animals in spite of the ubiquitous expression of #015 (*rpS3*), whereas there is a possibility that expression of #015 (*rpS3*) was merely affected by X-ray irradiation in *D. japonica*.

Two of the HiCEP clones showed a Type 1 expression pattern, in which cells expressing the genes were distributed in the mesenchymal space from in front of the eyes to the tail. One of them, #001 (unknown), showed homology with *NB.32.1g*, which is a novel gene identified by microarray analysis in *S. mediterranea* (Eisenhoffer *et al.*, 2008). Based on disappearance of the neoblasts followed by cells expressing *NB.32.1g* after X-ray irradiation, and cell tracing experiment using BrdU, it has been

proposed that *NB.32.1g*-expressing cells should be precursor cells of some differentiated cells (Eisenhoffer et al., 2008). It has also been reported that *p53* in *S. mediterranea* is involved in the production of cells expressing *NB.32.1g* (Pearson and Sánchez Alvarado, 2010). Another Type 1 clone was #038 (*p53*), indicating that cells expressing Type 1 clones are coincident with cells expressing *NB.32.1g* in *S. mediterranea*.

#### Heterogeneity of the neoblasts revealed by FBSC-PCR

In the case of ES cells, heterogeneous expression of *nanog*, which has an important role in maintaining the pluripotency of ES cells, is observed (Chambers et al., 2007). Nanog-expressing ES cells can keep their status as ES cells, whereas Nanog-negative ES cells undergo differentiation or return to the ES cell state (Chambers et al., 2007). Transitions of gene expression along the changing cell status are also observed in hematopoietic stem cells (Wilson et al., 2007). Furthermore, during neurogenesis in fly, expression levels of transcription factors is sequentially changed along the progression of embryogenesis, and these changes are required for proper neural cell production (Isshiki et al., 2001). Thus, manifestation about heterogeneity in a cell population at the molecular and single cell levels will bring us important information to define the cell state.

Classically, the neoblasts were considered to be a unique and homogeneous cell population. However, recent molecular research has demonstrated the heterogeneity of the neoblast population in *D. japonica*. *Djpiwi-1*, a piwi family gene in *D. japonica*, is expressed in the subpopulation of neoblasts located along the longitudinal dorsal midline (Rossi et al., 2006). *nanos* is expressed in the germline precursors in asexual planarians in *D. japonica* (Sato et al., 2006). The cells expressing *nanos* have stem cell features morphologically and express several neoblast specific genes (Sato et al., 2006; Yoshida-Kashikawa et al., 2007), meaning they are a subpopulation of neoblasts. This result was confirmed by the results of FBSC-PCR analysis, in which *nanos*-positive cells were found in the X1 fraction, a certain population among *DjpiwiA*-expressing cells (Hayashi et al., 2010). FBSC-PCR analyses also showed heterogeneity of the neoblasts based on the expression of several genes (Hayashi et al., 2010). Furthermore, FBSC-PCR revealed that genes related to DNA replication are predominantly expressed in the S-phase neoblasts, suggesting complex heterogeneous gene expression in the neoblast population (Hayashi et al., 2010). Here, we also found heterogeneous gene expression in the HiCEP clones (Fig. 5). #012 (*P2X-A*) belonging to Type 2A was expressed in about half of the neoblasts positive for *DjpiwiA* in the X1 fraction. In the X2 fraction, #012 (*P2X-A*)- and #008 (*piwiA*)-double positive cells were also found. #012 (*P2X-A*) encodes an ionotropic purinergic receptor, named *DjP2X-A*, and functions in maintaining the steady state of cell proliferation of the neoblasts (Sakurai et al., 2012). Although cells positive for *DjP2X-A* were distributed throughout the body along the anterior-posterior and medio-lateral axis like those positive for *DjPiwiA*, the *DjP2X-A*-expressing neoblasts were localized in the outermost layer of the mesenchymal space whereas *DjPiwiA* single positive neoblasts were observed in a more inner layer, reflecting heterogeneous expression as defined by FBSC-PCR. We do not know the details of regulatory mechanism of *DjP2X-A* expression in the neoblasts, but it might be related to be proliferation state.

## Conclusion

In this work, we surveyed planarian gene expression by HiCEP genome widely, and analyzed the expression of the genes by *in situ* hybridization, qPCR and single cell RT-PCR. By these multiple gene expression analyses, we could obtain at least 110 gene fragments as candidates, define at least 42 neoblast or related cell-specific genes classified into Type 1, Type 2A and B, and could reveal complexity of the gene expression in the neoblast population. These multidimensional data can provide important information not only about planarians, but also about stem cell biology.

The combination of HiCEP and *in situ* hybridization identified several kinds of neoblast-specific genes including genes predicted to be involved in chromatin modification, gene transcription, cell cycle, apoptosis and cell-cell interaction via the cell membrane as well as RNA metabolism. This sequence information expands our knowledge of molecular fundamentals in planarian stem cells. Through functional analyses of these genes in the future, we will be able to interconnect several molecular cascades in overall neoblast regulation. Furthermore, this gene set of the neoblasts in *D. japonica*, in combination with previous information (reviewed by Shibata et al., 2010), can be used to reveal common features of stemness across species by comparing gene sets among organisms. The data from the combination of *in situ* hybridization and FBSC-PCR will help us to understand the relationship between gene function and nonuniformity of the neoblasts. Also, we can utilize this method for analysis that defines the gene function involved in commitment of the neoblasts to restricted cell lineages after we identify precise cell markers. Thus, these multiple gene expression analyses are very useful for stem cell research not only in planarians, but also in other organisms, and might provide significant information for stem cell biology.

## Materials and Methods

### Animals

The asexual planarians of a clonal strain, SSP (sexualizing special planarian), of *D. japonica* were used. Planarians were kept in autoclaved tap water at 24°C. They were fed chicken liver every 2 weeks for maintenance. Planarians were starved for 7-14 days prior to experiments. About 8-mm-long planarians were sacrificed for the neoblast collections by FACS and FBSC-PCR, and up to 6-mm planarians were used for *in situ* hybridization.

### RNA preparation

Total RNA from the neoblasts collected by FACS was prepared using an RNeasy Mini kit (QIAGEN) for HiCEP. Total RNAs from intact and X-ray-irradiated planarians were prepared by the CsCl cushion method for HiCEP. Total RNAs from intact and X-ray-irradiated animals were prepared using ISOGEN-LS (ISOGEN-LS, Tokyo, Japan) for qPCR.

### X-ray irradiation

X-ray irradiation was performed as previously described (Yoshida-Kashikawa et al., 2007). Planarians placed on wet filter paper on ice were irradiated with 160 cGray (cGy) of X-rays using an X-ray generator (SOFTX B-4).

### HiCEP

HiCEP analysis was performed as described by Fukumura et al. (2003). Five micrograms of total RNA from each sample was used for first strand cDNA synthesis using a Superscript™ First-Strand Synthesis System (Invitrogen) with 100 pmol of 5' biotinylated oligo(dT) primer. After synthesis of second strand cDNA (dscDNA), the dscDNA was digested with MspI

(Takara, Ohtsu, Japan), followed by MspI adaptor ligation with T4 DNA ligase (NEB, Beverly, MA). The products ligated with poly(A)-biotin were collected using magnetic beads coated with streptavidin, Dynabeads M-280 streptavidin (Dyna, Oslo, Norway). After washing, the products on the beads were digested with MseI (NEB). The supernatant containing digested fragments was collected, followed by ligation to 10.2 pmol MseI adaptor using T4 ligase.

Using those adaptor-ligated dscDNA fragments, selective PCR based on AFLP was carried out. The 16 sequences of MspI-NN primers conjugated with fluorescent dye and the 16 sequences of MseI-NN primers were used. The PCR conditions were as follows: 95.0°C for 1 min, 28 cycles at 95.0°C for 20 sec, 71.5°C for 30 sec and 70°C for 1 min, followed by 60.0°C for 30 min. The PCR products were loaded on an ABI Prism 310 (Applied Biosystems, Foster City, CA) for electrophoresis. The data analysis was performed using GeneScan 3.1.2 (Applied Biosystems).

The aliquot of HiCEP product was loaded on a 10 % polyacrylamide gel. After silver staining of the gel, appropriate bands matching fragments of interest were cut out. The gel slices were suspended in 50 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), and 2 µl of supernatant was used for PCR.

#### Whole-mount *in situ* hybridization

All digoxigenin-labeled RNA probes were prepared using pBlue-scriptSK(-) vectors containing the insert from the EST clone or pCR II-TOPO (Invitrogen) vector containing PCR product and a digoxigenin-labeling kit according to the manufacturer's instructions (Roche). Manual whole-mount *in situ* hybridization using alkaline phosphatase and 4-nitroblue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) color development was carried out as described by Umesono *et al.*, 1997. Also automatic *in situ* hybridization was carried out by InsituPro (ABIMED, Langenfeld, Germany).

#### Quantitative reverse transcription-polymerase chain reaction

First-strand cDNAs complementary to total RNA from intact and X-ray-irradiated planarians were synthesized using a QuantiTect, Reverse Transcription kit (QIAGEN). Diluted cDNAs (20X) were used for semi-quantitative reverse transcription-polymerase chain reaction (qPCR). Ten microliters of qPCR mixture was composed of 1X Quantitect SYBR green PCR master mix (QIAGEN), 0.3 µM gene primers and diluted cDNA, and incubated in an ABI PRISM 7900 HT (Applied Biosystems). The reaction conditions were as follows: 50°C for 2 min, 95°C for 15 min, 50 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 1 min. To check the products, 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec was added to determine the melting curve at the end of the reaction.

#### Single cell RT-PCR

Single cell gene expression analyses using FACS-based single cell RT-PCR (FBSC-PCR) were performed as described by Hayashi *et al.*, (2010). Gene specific primers were as follows:

##### DjG3PDH

FW: ACCACCAACTGTTTAGCTCCCTTAG  
RV: GATGGTCCATCAACAGTCTTTTGC

##### DjMHC-A

FW: TCTGAAGAAAGAGCTGATCAAGCTGAACAA  
RV: TGGATTACAGTAGTCGTACCAGGTGCCA

##### Djsyt

FW: GAACTCCGGCATTGGTCCGGA  
RV: GTTAAGCTTTTGTTCATTAATTGTTTTT

##### #001 (unknown)

FW: TATGATGAAAATGATAAAGCAACACGAA  
RV: CATTCTTTATCCAGTTTTTTTACTCCATT

##### #004 (unknown)

FW: AATTATTGCTTGCGATGGTCTCTG  
RV: TATCTCCGCTCATTTTCAATACGG

##### #007 (DjSWI/SNFr)

FW: AAAAATGTGGGAGGCATGTAAAGAC  
RV: CTTCTCTTCATGATTACCATTGACC

##### #008 (piwiA)

CGAATCCGGGAAGTGTCTGATG  
RV: GGAGCCATAGGTGAAATCTCATTTG

##### #009 (PRMT)

FW: AGTCAATAACGGTGAAGAGATAACTGG  
RV: CCTTCCGACCTACCTCATTCG

##### #012 (P2X-A)

FW: GATTTCAACAATGGAATGAATTTTAGATA  
RV: AAAATGTGAAACAAGTAGCAGGATCA

##### #015 (rpS3)

FW: TGACGGATTAATGATTCACAGTGG  
RV: TGAATCAGGCAAAGGACGC

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