

Diverse miRNA spatial expression patterns suggest important roles in homeostasis and regeneration in planarians

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ABSTRACT miRNAs are an important class of non-protein coding small RNAs whose specific functions in animals are rapidly being elucidated. It is clear that miRNAs can play crucial roles in stem cell maintenance, cell fate determination and differentiation. We use planarians, which possess a large population of pluripotent somatic stem cells, as a powerful model system to study many aspects of stem cell biology and regeneration. In particular we wish to investigate the regulatory role miRNAs may have in planarian stem cell self renewal, proliferation and differentiation. Here, we characterized the differential spatial patterns of expression of miRNAs in whole and regenerating planarians by *in situ* hybridization to nascent miRNA transcripts. These miRNA expression patterns are the first which have been determined for a Lophotrocozoan animal. We have characterized the expression patterns of 42 miRNAs in adult planarians, constituting a complete range of tissue specific expression patterns. We also followed miRNA expression during planarian regeneration. The majority of planarian miRNAs were expressed either in areas where stem cells (neoblasts) are located and/or in the nervous system. Some miRNAs were definitively expressed in stem cells and dividing cells as confirmed by *in situ* hybridisation after irradiation. We also found miRNAs to be expressed in germ stem cells of the sexual strain. Together, these data suggest an important role for miRNAs in stem cell regulation and in neural cell differentiation in planarians.

KEY WORDS: *planarian, miRNA, expression, Schmidtea mediterranea, regeneration*

Introduction

The discovery of miRNAs uncovered an underlying method of gene regulation that was almost completely unknown little over a decade ago. miRNAs are an abundant class of non-protein-coding small RNAs (Lee *et al.*, 1993) that may collectively be the largest group of regulatory genes in animal genomes. Since their ubiquity was first demonstrated in 2001 (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001), thousands of miRNAs have been identified in animals, plants and viruses (Griffiths-Jones *et al.*, 2006), with many of them being evolutionarily conserved (Pasquinelli *et al.*, 2003; Prochnik *et al.*, 2007). Recent studies suggest that miRNAs regulate gene expression in more than 30% of protein coding genes in humans and other animals (Berezikov *et al.*, 2005). miRNAs regulate gene expression by exact complementarity dependent cleavage of the tar-

geted mRNA in plants (but rarely in animals), and by inhibiting translation through imperfect complementarity to target sites in animal genes (Lai, 2002; de Moor *et al.*, 2005; Robins and Press, 2005; Stark *et al.*, 2005; Sun *et al.*, 2005). In this way, miRNAs regulate multiple biological processes, including developmental timing (Reinhart *et al.*, 2000), stem cell division (Houbaviy *et al.*, 2003; Hatfield *et al.*, 2005), apoptosis (Cheng *et al.*, 2005; Cimmino *et al.*, 2005) and their inappropriate activity contributes to disease (Álvarez-García and Miska, 2005), including cancer (Meltzer, 2005).

miRNAs have previously been described in planarians (Palakodeti *et al.*, 2005), including both conserved miRNAs present in other phyla as well as some that may be specific to

Abbreviations used in this paper: LNA, locked nucleic acid; miRNA, microRNA.

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planarians. Planarians are bilaterally symmetrical metazoans, members of the phylum platyhelminthes within the major protostome clade, the lophotrochozoa (Carranza *et al.*, 1997; Dunn *et al.*, 2008). Planarians are well known for their incredible regenerative capacity (Morgan, 1900); tiny fragments of their bodies can regenerate into an entire animal perfectly scaled to the new size. Planarian tissues contain several differentiated non-proliferating cell types, with all mitotically active cells currently grouped together as neoblasts. Neoblasts reside in the parenchymal tissue and are believed to be pluripotent somatic stem cells which through proliferation and correct differentiation are responsible for the regenerative capacity of planarians. Together with their immediate progeny, neoblasts account for approximately 20-30% of all parenchymal cells (Baguña, 1976). The proliferative capacity and demonstrable multi-potency of the neoblast cell population allow planarians to have extreme tissue plasticity, which is apparent not only in their regenerative capacity but also in their ability to remodel their body pattern indefinitely in response to rounds of starvation and feeding (Saló, 2006).

Palakodeti *et al.* (2005) reported the cloning of 71 miRNAs from the planarian *Schmidtea mediterranea*. Given the fact that in other organisms miRNAs have been implicated in the control of stem cell function, cell lineage decisions and cell differentiation, it seems likely that miRNAs play a central role in regulating regeneration and normal homeostatic turnover in planarians.

Although whole-mount *in situ* hybridization (ISH) is a powerful technique for detecting gene expression in planarians (Umesono *et al.*, 1997; Sánchez Alvarado *et al.*, 2002; González-Estévez *et al.*, 2007), sub-cellular localization of transcripts has not been previously described in these organisms. For instance, in *Drosophila*, typical mRNAs are predominantly observed in the cytoplasm but primary miRNA transcripts have been detected as nascent transcripts at each chromosomal locus in the nucleus (Kosman *et al.*, 2004; Ronshaugen and Levine, 2004). This approach seems to have broader success than others, in *Drosophila* at least, and circumvents the high cost of approaches using Locked Nucleic Acids (LNA) (Aboobaker *et al.*, 2005; Wienholds *et al.*, 2005). We have used this approach to examine the expression of *Schmidtea mediterranea* miRNAs. We clearly show that the sub-cellular localization of planarian mRNAs is predominantly in the cytoplasm, but nascent transcripts can also be faintly detected in the nuclei. Use of long probes against the primary miRNA transcript detects transcripts exclusively in the nucleus while detection with LNA probes shows exclusive localization in the cytoplasm. We see the full spectrum of tissue specific and expression patterns described for other planarian genes. Although we detect miRNAs in all tissues we find that the majority of planarian miRNAs have enriched expression in the central nervous system (CNS) and in the parenchyma. Some conserved miRNAs also have expression patterns that may be conserved with respect to their tissue specific expression in other bilaterians, including some stem cell-related miRNAs. These expression patterns suggest possible conserved functions in both stem cell and germ stem cell regulation for phylogenetically conserved miRNAs for which functions are not yet known in other organisms. Finally, we show that most planarian miRNAs are differentially regulated during regeneration. Overall our data suggest an important role of planarian miRNAs in stem cell regulation, stem cell differentiation during regeneration, neural regeneration and dif-

ferentiation and pattern maintenance.

Results

Design of primary miRNA in situ probes in S. mediterranea

It has previously been shown that short conventional nucleic acid probes corresponding to the pre-miRNA or to the mature miRNA sequences fail to give signals but that longer probes to the primary transcript of the pri-miRNA can detect nascent miRNA transcripts (Aboobaker *et al.*, 2005). We generated long 400-700bp digoxigenin-labeled antisense genomic probes to all known *Schmidtea mediterranea* miRNA loci including the putative planarian specific miRNAs (Palakodeti *et al.*, 2006) (Supplementary Table) and hybridized them as described in *Materials and Methods*. Where a few miRNAs resided in a small cluster, as is the case for: sme-mir-2a-1/sme-mir-71a-1, sme-mir-2a-2/sme-mir-71a-2, sme-mir-2c/sme-mir-71c, sme-mir-277c/sme-mir-277d, sme-mir-750/sme-mir-755, we designed only one probe to span each cluster. When multiple miRNAs resided in a long cluster we designed non-overlapping sub-cluster probes for the following clusters: sme-mir-277a/sme-mir-277b (two probes) and the sme-mir-2d/sme-mir-71b/sme-mir-752 cluster (three probes, one including sme-mir-2d, sme-mir-71b and sme-mir-752, another which included sme-mir-71b and sme-mir-2d and the last including sme-mir-752). sme-mir-13 had been assigned to this cluster (Palakodeti *et al.*, 2006) but the stem-loop sequence we found in the *S. mediterranea* genome trace archive (<http://genome.wustl.edu>) had several mismatches compared to the described sequence, so we excluded it from our analyses. This is consistent with the fact that Palakodeti *et al.* (2006) were not able to clone this miRNA in their extensive screen.

In designing our probes we attempted to minimize the possibility of including host gene exons for those miRNAs that may be intronic (The current *S. mediterranea* genome assembly does not allow for this to be done unambiguously). For corroborating that the probe templates did not include the host gene exons we performed BLAST searches and looked for predicted open reading frames in our probe template sequences and the regions surrounding them. We found no putative open reading frames longer than 100 bp within our probes or for 300 bp either side on the same strand as the miRNAs suggesting that our probes are likely to be all non-coding. More significantly none of our templates had significant BLAST similarity to any known gene (E-Value ≥ 0.1) except for the antisense strand to sme-mir-C probe template sequence. This miRNA is embedded in the antisense strand of an *S. mediterranea* gene with homology to *Djcbc-1* (E-Value $9e-09$), a DEAD box RNA helicase of the RCK/p54/Me31B family in *Dugesia japonica*. The expression pattern of this DEAD box RNA helicase in *S. mediterranea* (data not shown) was the same pattern as that known for *D. japonica* (Yoshida-Kashikawa *et al.*, 2007) and different to the expression pattern of the strand giving rise to sme-mir-C (see below).

We also performed a BLAST (blastn, tblastn) against all the planarian ESTs available from NCBI. sme-mir-61 was the only miRNA present in ESTs (E-value 0; NCBI Accession numbers: DN302088, DN308595, DN298481, DN306489), corresponding to a gene of unknown function without a clear open reading frame. Finding pri-miRNA transcripts in smaller EST collections is rare, but nonetheless pri-miRNAs do appear among EST collections.

TABLE 1

GROUPING OF THE DIFFERENT EXPRESSION PATTERNS OF PLANARIAN miRNAs

Group	miRNA	Adult expression pattern	Sexual strain	Irradiation sensitive	
1	sme-mir-2b	digestive system (10/15)		not determined	
	sme-mir-10	digestive system (11/15)		not determined	
2	sme-mir-Ea	digestive system, brain ganglia (14/15)		not determined	
3	sme-mir-749	epidermis (24/24)		No	
4	sme-mir-1a	excretory system, parenchyma (15/19)		No	
	sme-mir-C	excretory system, parenchyma (25/25)		?	
5	sme-mir-1c	brain ganglia, nerve cords (14/15)		not determined	
	sme-mir-31b	brain ganglia, nerve cords enhanced posteriorly (34/34)		No	
	sme-mir-124c	entire CNS (nerve cords enhanced posteriorly) (11/15)		not determined	
6	sme-mir-87b	brain ganglia, pharynx (34/34)		No	
7	sme-mir-7c	brain ganglia, nerve cords, parenchyma including midline (15/15)		?	
8	sme-bantam-a	medial parenchyma, partial posterior midline (15/15)	Sexual/germline (4/6)	not determined	
	sme-lin-4a	medial parenchyma, partial posterior midline (18/19)		No	
	sme-lin-4c	medial parenchyma, partial posterior midline (19/19)		No	
	sme-mir-7a	medial parenchyma, partial posterior midline (15/15)		not determined	
	sme-mir-277c/277d	medial parenchyma, partial posterior midline (15/15)		not determined	
9	sme-bantam-b	medial parenchyma, brain ganglia (15/15)	Sexual/germline (4/6)	not determined	
	sme-bantam-c	medial parenchyma, brain ganglia (15/15)		not determined	
	sme-let-7b	medial parenchyma, brain ganglia (24/24)		No	
	sme-mir-1b	medial parenchyma, brain ganglia (13/15)		not determined	
	sme-let-7c	medial parenchyma, partial posterior midline, brain ganglia (19/19)		Yes	
	sme-mir-2a-1/71a1	medial parenchyma, partial posterior midline, brain ganglia (19/19)		No	
	sme-mir-2a-2/71a2	medial parenchyma, partial posterior midline, brain ganglia (17/19)		No	
	sme-mir-2c/71c	medial parenchyma, partial posterior midline, brain ganglia (19/19)		Yes	
	sme-mir-67	medial parenchyma, partial posterior midline, brain ganglia (18/19)		Sexual/germline (4/6)	Yes
	sme-mir-79	medial parenchyma, partial posterior midline, brain ganglia (15/15)		Sexual (6/6)	not determined
	sme-mir-281	medial parenchyma, partial posterior midline, brain ganglia (16/19)			No
	sme-mir-745	medial parenchyma, partial posterior midline, brain ganglia (18/19)			No
	sme-mir-746	medial parenchyma, partial posterior midline, brain ganglia (14/15)			not determined
	sme-mir-747	medial parenchyma, partial posterior midline, brain ganglia (17/19)			?
sme-mir-2d/71b/752	parenchyma, brain ganglia (19/19)	Sexual* (6/6)	Yes		
sme-mir-7b	parenchyma, brain ganglia (19/19)		Yes		
sme-mir-36	parenchyma, brain ganglia (17/19)	Sexual/germline (5/6)	Yes		
sme-mir-92	parenchyma, brain ganglia (16/19)		Yes		
sme-mir-124a	parenchyma, brain ganglia (19/19)		Yes		
sme-mir-124b	parenchyma, brain ganglia (19/19)		Yes		
10	sme-mir-756	not determined	Sexual/germline (6/6)	not determined	

The table shows the 9 classes of expression patterns found in adult asexual planarians and the one only (number 10) expressed in the sexual strain. Different subgroups inside a group are differentially coloured. The numbers in the columns of "adult expression pattern" and "sexual strain" refer to the number of planarians showing that specific expression pattern from the total of planarians used for the ISH in at least two independent experiments. When the proportion is not 100% ISH in some worms did not work for technical reasons or because some worms in a batch failed to stain. For the sexual strain this was invariably because some planarians were not reached complete sexual maturity. Bold numbers highlight those miRNAs where significant expression pattern variation was observed. This may be due to technical reasons or more likely reflect real homeostatic differences among the animals. These variations always show some of the specific features of the miRNA expression pattern. The tag "Sexual" means that previous work cloned more copies of a particular miRNA in the sexual strain than in the asexual strain of *S. mediterranea* (Palakodeti *et al.*, 2006); "germline" means that our work shows that the miRNA is expressed in the germline. From the sme-mir-2d/71b/752 locus only sme-mir-2d was cloned more times in the sexual strain than in the asexual one (Palakodeti *et al.*, 2006), indicated by an asterisk in the table. In the "Irradiation sensitive" column, "Yes" in red means that some staining remains at the beginning of the posterior midline (area A in Supplementary Fig. 8). A question mark means that it was not possible to unambiguously clarify if all parenchymal staining was lost in irradiated worms.

These analyses suggest our probes will detect *bona fide* nascent transcripts including the miRNA hairpin sequences of interest, rather than cytoplasmic mRNAs that contained miRNA sequences in their introns.

Spatial expression patterns of miRNAs in adult asexual planarians

We performed whole-mount *in situ* hybridization (ISH) for all known planarian miRNAs (see Supplementary Table, Table 1, Fig. 1 and Supplementary Fig. 1). ISH for every miRNA was performed in at least 3 independent experiments and with a total of at least 5 planarians per miRNA per experiment (Table 1). For some miRNAs we noted a consistent variation in ISH patterns. In particular around 1/3 of the animals had alternative expression patterns for sme-mir-31b and sme-mir-C (Supplementary Fig. 2). We believe this may be in part due to technical reasons but may also reflect real homeostatic variation in the expression patterns of these miRNAs.

Identical expression patterns were always generated using different probes to the different miRNAs of a miRNA cluster, consistent with coordinate expression of these clustered miRNAs as single primary transcripts (see example of cluster sme-mir-2d/sme-mir-71b/sme-mir-752 in Supplementary Fig. 1W, X). From a total of 71 miRNAs reported in planarians (Palakodeti *et al.*, 2006) we obtained 36 patterns in asexual adult planarians, which represents more than half (41/71) of the known miRNAs in planarians.

We found clear examples of miRNAs expressed in a selection of tissue specific patterns and tissue specific combinations (Fig. 1 and Supplementary Fig. 1) including: digestive system (2/41); digestive system and nervous system (1/41); epidermis (1/41); excretory system and/or gland cells and parenchyma (2/41); nervous system (3/41); nervous system and pharynx (1/41); strong nervous system and parenchyma (1/41); parenchyma (8/41); and strong parenchyma expression with weaker nervous system expression (22/41).

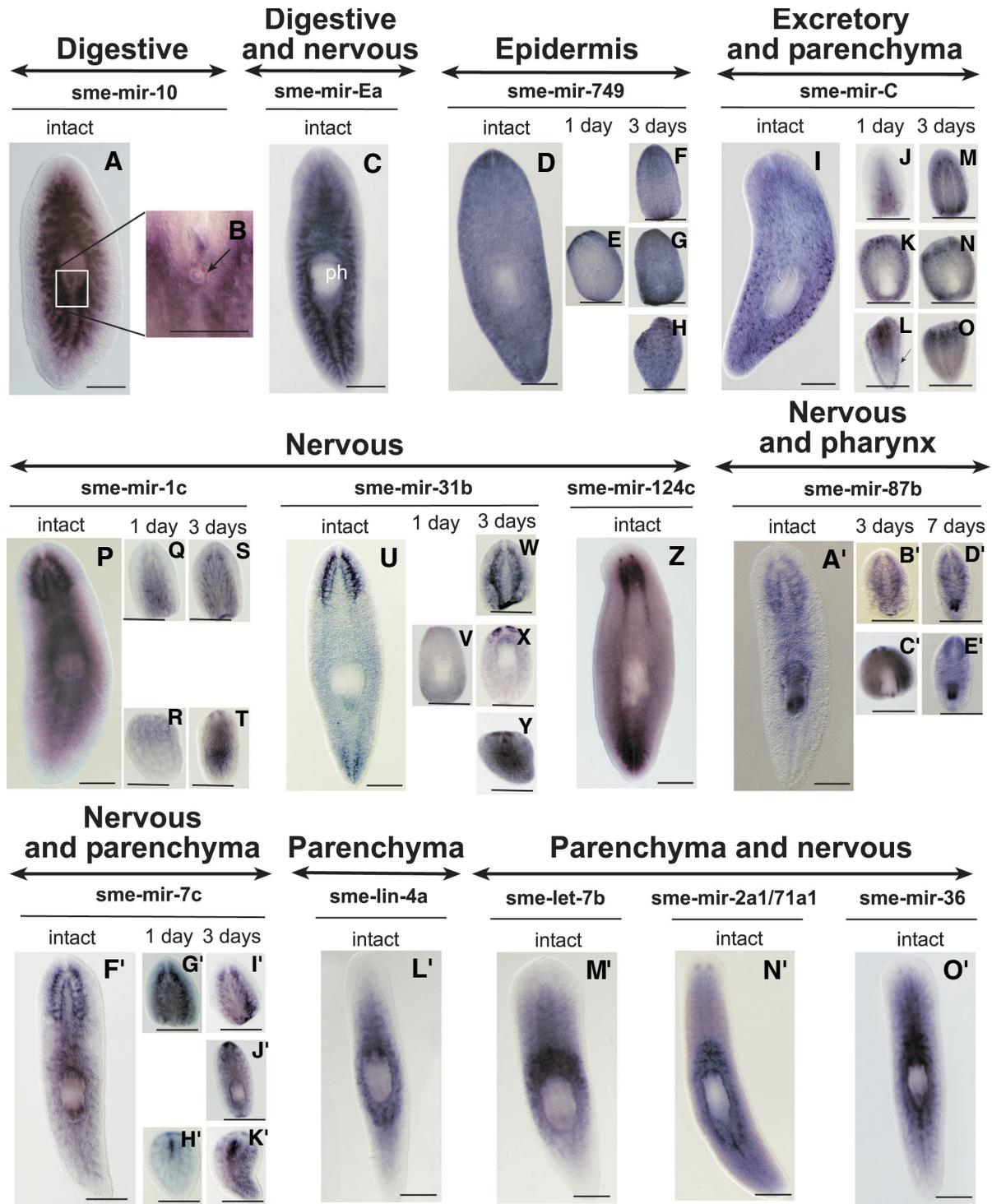


Fig. 1. Planarian miRNA expression patterns in asexual intact and regenerating *S. mediterranea*. Whole-mount ISH of the most representative miRNAs in intact and/or regenerating planarians. The different expression patterns can be classified into 9 classes. Representative miRNAs whole-mount ISH corresponding to each of the 9 classes are presented (see Table 1). For the regenerating fragments, the levels of amputation are always anterior and posterior to the pharynx (ph). (A,B) *sme-mir-10* showing expression in the entire digestive system and in the opening of the pharynx; B shows high magnification of the opening of the pharynx (arrow). (C) *sme-mir-Ea* showing expression in the posterior branches of the digestive system and in the brain ganglia. (D) *sme-mir-749* showing expression in the epidermis. During regeneration its expression is up-regulated in the wound at 1 day of regeneration (E). At 3 days of regeneration expression has increased in the epidermis around the blastema (F-H). (I) *sme-mir-C* showing expression in the parenchyma, the more posterior part of the excretory system and the subepidermal marginal adhesive gland cells. It is clearly up-regulated at 3d of regeneration at the postblastema level (M-O) but not at 1 day of regeneration (J-L) with exception of regenerating tails which present a clear up-regulation in the supepidermal marginal adhesive gland cells (L, see arrow). miRNAs with expression in the CNS are represented by *sme-*

Over 70% (31/41) of the miRNAs have some expression in the nervous system. The planarian CNS consists of two cephalic ganglia situated above two ventral nerve cords (VNCs) that run along all the body of the animal that are connected by transverse commissures (Reuter and Gustafsson, 1995; Cebrià *et al.*, 2002). Most miRNA expression seems to be found surrounding the cephalic ganglia or the cephalic ganglia and the VNCs (Fig. 1). According to previous classification of nervous markers in planarians (Nakazawa *et al.*, 2003), this would correspond to class B genes, which are suggested to be involved in signal transduction to the VNC. An alternate view is that these expression patterns are labelling cells destined or committed to differentiate into cells of the CNS as older neurons die, implicating miRNAs in the process of neural differentiation. One exception to this peri-neural expression is the pattern of sme-mir-124c (Fig. 1Z), which, like its homolog in *Drosophila*, is expressed in the whole CNS (Aboobaker *et al.*, 2005). In contrast expression of mir-124 is limited to the developing brain in vertebrates (Lagos-Quintana *et al.*, 2002). Other conserved nervous-related expression patterns with respect to *Drosophila* embryogenesis (Aboobaker *et al.*, 2005), can be seen for sme-mir-7c (Fig. 1F') and sme-mir-2c (Supplementary Fig. 1L), sme-mir-7b and sme-mir-92 (Supplementary Fig. 1S, V).

Perhaps most significantly more than 80% (34/41) of the miRNAs have some parenchymal expression, including the entire or partial midline. The planarian parenchyma is a mesodermal tissue with 20-30% of the cells in this space being putative neoblasts (Baguña, 1976). Many of the parenchymal patterns (15/28) are specific to the parenchymal region which corresponds to the medial regions of the body surrounding the pharynx and to the more anterior part of the midline posterior to the pharynx (Fig. 1L', N' and Supplementary Fig. 1C-F, J-R). 7 out of 28 miRNA expression patterns (Fig. 1F', O' and Supplementary Fig. 1S-X) show the typical expression pattern of proliferating neoblasts in the parenchyma (Salveti *et al.*, 2000; Orii *et al.*, 2005; Reddien *et al.*, 2005a; Salvetti *et al.*, 2005; Guo *et al.*, 2006; Rossi *et al.*, 2006). A variation on this parenchymal expression pattern is seen for 4 out of 28 parenchymally expressed miRNAs (Fig. 1M' and Supplementary Fig. 1G-I). These miRNAs sme-let-7b, sme-bantam-b, sme-bantam-c and sme-mir-1b have expression which is just restricted to the more anterior medial parenchyma and lack expression in the posterior.

Only a few miRNAs are expressed in areas outside of the parenchyma or nervous system. The planarian digestive system is formed by a pharynx and a blind gut with three major branches (two posterior and one anterior) that diverticulate into more lateral

tissues and lacks a separate anus (Saló, 2006). 3 miRNAs are expressed in the digestive system, sme-mir-10, sme-mir-Ea and sme-mir-2b. miR-10 expression is detected in the hindgut, posterior primordial midgut and in the anal pad in fully germband-retracted *Drosophila* embryos (Aboobaker *et al.*, 2005). sme-mir-10 (Fig. 1A, B) is expressed in the entire gut and in the opening of the pharynx, suggesting there may be some conservation of mir-10 function in gut tissues across protostome phyla. sme-mir-Ea is only expressed in the posterior part of the digestive system, in addition to the brain ganglia (Fig. 1C). sme-mir-2b (Supplementary Fig. 1A) is expressed in the entire digestive system and in the pharynx. One miRNA, sme-mir-87b, has part of its expression pattern in the pharynx (Fig. 1A') and sme-mir-749 is expressed in cells at or near the surface of the animal (Fig. 1D and Supplementary Fig. 3), in a similar way to EST clone NCBI: 32899321 (AHNAK-related protein) in *Dugesia japonica* (Rossi *et al.*, 2007). The excretory system is a dorsal network of canals with specialized flame cells (Saló, 2006). We found that sme-mir-C (Fig. 1I), a specific planarian miRNA and sme-mir-1a (Supplementary Fig. 1B) are expressed only in the mid-posterior part of the excretory system, in sub-epidermal marginal adhesive gland cells (Sánchez Alvarado *et al.*, 2002) and in the entire parenchyma.

Expression patterns of miRNAs in regenerating planarians

In order to investigate whether miRNAs play any role during planarian regeneration, we performed whole-mount ISH on regenerating planarians. We used 1 day (a stage when neoblast proliferation is taking place), 3 day (when differentiation is proceeding) and 7 day (when all new structures are essentially in place) regenerating planarian pieces. ISH for every miRNA and regeneration timepoint was performed in at least 2 independent experiments, with a total of at least 3 planarians per miRNA and per regeneration timepoint. Planarians were amputated anterior and posterior to the pharynx and fixed at the appropriate time point.

We observed clearly distinct expression patterns for most of the miRNAs that gave expression in non-regenerating planarians.

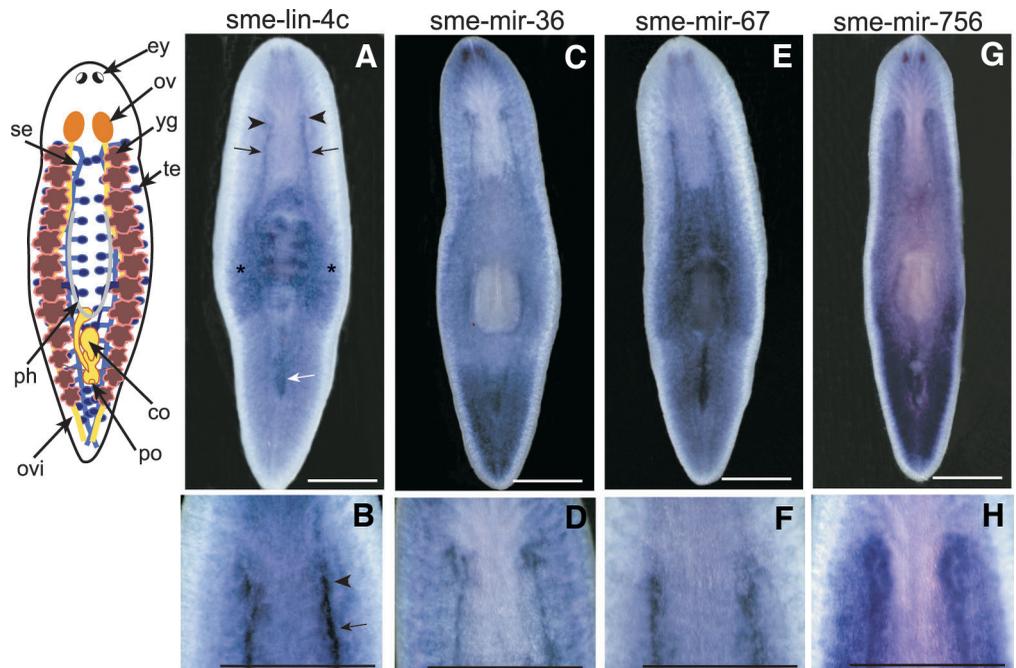
sme-mir-749 (Fig. 1D) is expressed at or very close to the epidermis of adult planarians (Supplementary Fig. 3). During regeneration, sme-mir-749 showed expression at or near the epidermis of the anterior and posterior blastemas in 1 day and 3 day regenerating pieces (Fig. 1E-H). sme-mir-C (Fig. 1I) is expressed in the mid-posterior part of the excretory system and sub-epidermal marginal adhesive gland cells and in the parenchyma. At 3 days of regeneration, sme-mir-C is expressed in the post-blastema (Fig. 1J-O). Interestingly, we found that the expression

(Legend continued from previous page)

mir-1c (P) showing expression surrounding the cephalic ganglia, by sme-mir-31b (U) showing expression surrounding the cephalic ganglia and the ventral nerve cord (VNC) enhanced anterior and posteriorly, and by sme-mir-124c (Z) which is expressed in the entire CNS. During regeneration, sme-mir-1c expression is not seen at 1 day (Q,R) but at 3 days weak expression occurs in the brain primordia and VNCs (S,T); sme-mir-31b is not up-regulated at 1 day of regeneration (V) but at 3 days is expressed at the in the new differentiating brain ganglia and VNC (W-Y). (A') sme-mir-87b which is expressed surrounding the cephalic ganglia and in the pharynx; during regeneration, up-regulation starts at 3 days in the primordium of the pharynx and in the new differentiating brain ganglia (B',C') and the same expression can be still observed at 7 days of regeneration (D',E'). (F') sme-mir-7c whose expression is shown surrounding the cephalic ganglia and in the parenchyma in adult organisms; at 1 day of regeneration sme-mir-7c is already expressed in the brain primordium and in the posterior midline (G',H') and this expression continues to 3 days (I'-K'). During posterior regeneration, up-regulation in the new differentiating VNCs starts at 3 days (I'). (L') sme-lin-4a which is expressed in the middle parenchyma and at the beginning of the posterior midline. miRNAs with parenchymal expression accompanied by weaker neural expression can be categorized into 3 subgroups all of them with a weak expression around the cephalic ganglia in addition to the parenchymal expression represented by (M') sme-let-7b, central parenchyma, (N') sme-mir-2a1/71a1 central parenchyma and the beginning of the posterior midline and (O') sme-mir-36 which is expressed in the parenchyma in pattern similar to other known neoblast markers. (A-H, P-G', I' L'-O') ventral views; (I-O, H', J'-K') dorsal views. Scale bars indicate 1 mm.

Fig. 2. Planarian miRNAs expressed in sexually mature planarians.

The diagram to the left shows the reproductive system of *Schmidtea mediterranea* sexual strain. The abbreviations indicate: co, copulatory apparatus; ey, eye; ov, ovary; ovi, oviduct; ph, pharynx; po, genital pore; se, seminal duct; te, testes; yg, yolk gland. *sme-lin-4c* (A), *sme-mir-36* (C), *sme-mir-67* (E) and *sme-mir-756* (G) whole-mount ISH showing expression in the ovaries (black arrow heads), oviduct (black arrows), yolk glands (black asterisk) and in the copulatory apparatus (white arrow). (B,D,F,H) High magnifications from the ovary (black arrow head) region showing part of the oviduct (black arrow). Note that expression of *sme-lin-4c* (B) has a localised dot at the base of the oviduct while D, F and H show more dispersed expression profiling the oval shape of the ovary. All images are in ventral position. Scale bars indicate 2 mm.



in the sub-epidermal marginal adhesive gland cells was enhanced in 1 day regenerating tails and that this expression disappeared after 3 days (Fig. 1L, O).

For all the miRNAs with predominantly neural expression in whole animals expression was only observed from 3 days of regeneration onwards in the primordial anterior and/or primordial posterior nervous system (for example *sme-mir-1c* (Fig. 1Q-T), *sme-mir-31b* (Fig. 1V-Y) and *sme-mir-87b* (B'-E')). The only exception is *sme-mir-7c*, where we could detect expression as early as 1 day of regeneration (Fig. 1H'). *sme-mir-7c* has strong expression around the brain and less prominent expression in the parenchyma including the midline of intact planarians. During anterior regeneration of tail pieces early neural expression was accompanied by high expression in the posterior midline (Fig. 1H', K'). For *sme-mir-87b*, where the expression is also in the pharynx of adult animals, as well as the brain, we observed expression of the miRNA in the primordial pharynx later in regeneration (Fig. 1B'-E').

For all the miRNAs with predominantly parenchymal expression patterns we observed more or less expression in the posterior midline in 3d regenerating planarians, similar to the pattern of *sme-mir-7c* during regeneration (Supplementary Fig. 4). This pattern is characteristic for neoblast markers (Salvetti et al., 2000; Rossi et al., 2006). In the case of those miRNAs where weak neural expression accompanied parenchymal expression the neural expression did not change during regeneration (Supplementary Fig 4).

Spatial expression patterns of miRNAs in mature sexual planarians

Palakodeti et al., 2006 observed differences in the number of times miRNAs were cloned from the sexual strain of *S. mediterranea* with respect to the asexual strain, suggesting differential miRNA expression between the strains. However, the authors could not observe quantitative differences by Northern blot analyses. In order to elucidate any differential miRNA spatial expression pat-

terns in sexual *S. mediterranea* compared to the asexual strain we performed whole-mount ISH on sexually mature *S. mediterranea* (Table 1). ISH for every miRNA was performed in at least in 2 independent experiments and with at least 3 sexually mature planarians per miRNA (Table 1).

Hermaphrodite *S. mediterranea* have a complex reproductive system formed by ventrally located ovaries posterior to the cephalic ganglia and two dorso-lateral lines of testes and yolk glands (Saló, 2006). Among all the tested miRNAs we could detect expression in the ovaries, oviduct, yolk glands and copulatory apparatus for *sme-lin-4c*, *sme-mir-36*, *sme-mir-67* and *sme-mir-756* (Fig. 2), with similar patterns to known markers of the planarian germline (Hase et al., 2003; Zayas et al., 2005; Handberg-Thorsager and Saló, 2007; Wang et al., 2007). Expression in the testes could not be unambiguously detected for any miRNAs, perhaps because this was masked by the higher background we sometimes observe with the nascent transcript ISH protocol we use or because the level of expression is not high enough for clear detection (Zayas et al., 2005). For the other miRNAs the expression in the sexual strain was equivalent to the asexual strain (Supplementary Fig. 5).

Planarian ovaries are round/oval structures organized with the oogonia at the base and periphery and the ova in the lumen of the ovary (Hyman, 1951; Handberg-Thorsager and Saló, 2007). Expression in the ovaries for *sme-mir-36*, *sme-mir-67* and *sme-mir-756* is slightly different to the expression of *sme-lin-4* (Fig 2). The latter seems to be expressed in the lumen of the ovary while the rest are expressed in the periphery where the undifferentiated germ cells or oogonia reside. This suggests that *sme-mir-36*, *sme-mir-67* and *sme-mir-756* are expressed in germ stem cells in planarians.

Some miRNAs are expressed in planarian stem cells

Neoblasts are defined as the only proliferating cells in planarians. Cell proliferation in planarians can be abrogated by lethal doses of gamma radiation (Dubois, 1949; Orii et al., 2005; Rossi et

al., 2007) as it results in the death of all dividing cells. This allows us to compare animals lacking neoblast stem cells with wild type animals. We took advantage of this approach in order to elucidate which miRNAs with parenchymal expression (suggestive of neoblast expression) are actually expressed in cells with proliferative capacity (Table 1).

Asexual planarians were irradiated at lethal doses of gamma radiation and fixed 6 days later when all cells with proliferative capacity have been lost. We were especially interested in the miRNAs which showed a similar pattern to other known neoblast markers (mir-752/2d/71b, sme-mir-7b, sme-mir-7c, sme-mir-36, sme-mir-92, sme-mir-124a and sme-mir-124b) and in those that showed expression in germ line associated cells in the sexual strain (sme-mir-36, sme-mir-67, sme-mir-756 and sme-lin-4c).

Of these miRNAs sme-mir-752/2d/71b, sme-

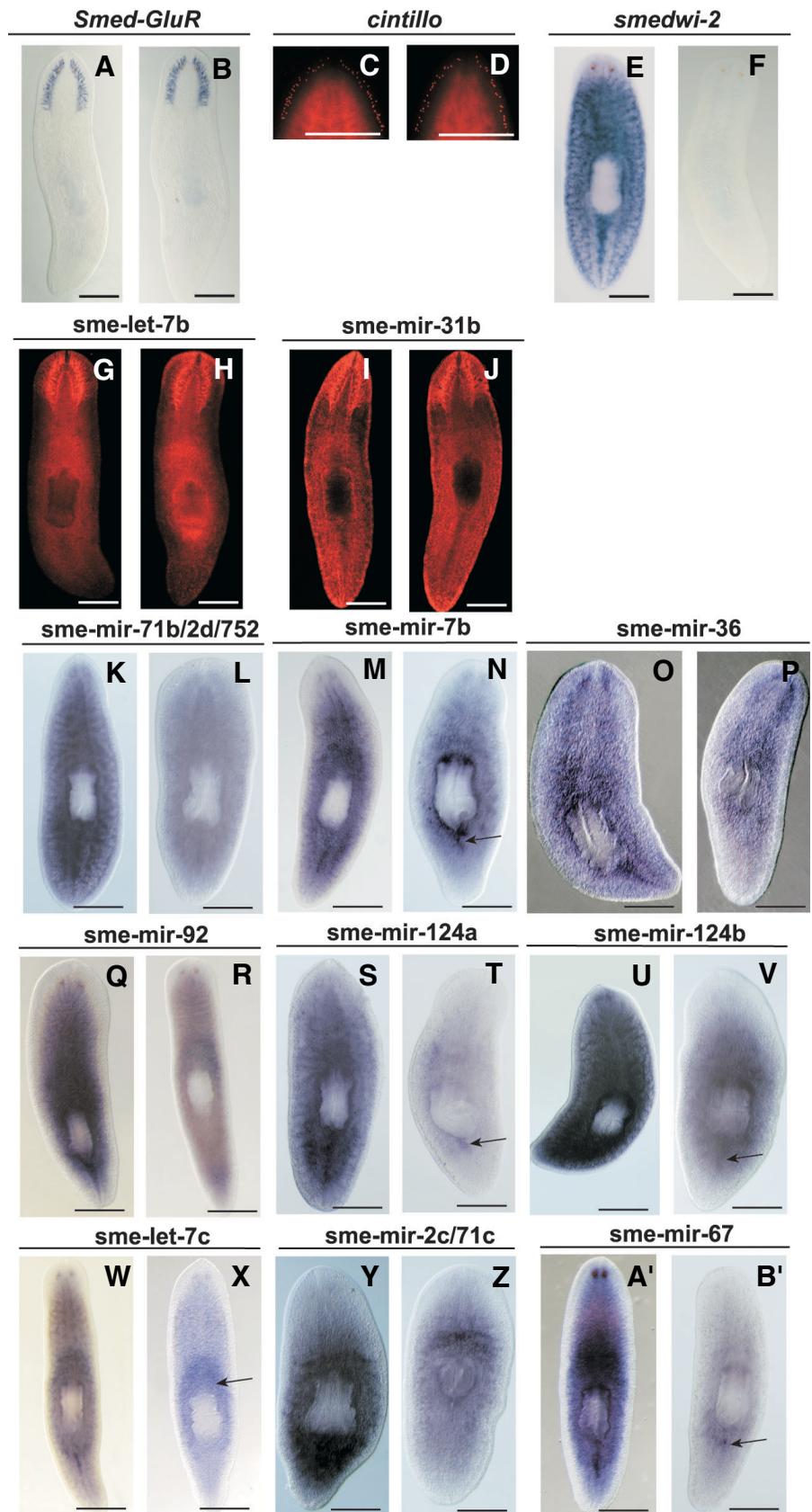


Fig. 3. Planarian miRNAs expressed in neoblasts.

The figure shows 5 controls versus the 9 miRNAs whose expression, analyzed by whole-mount ISH, is sensitive to high dose gamma radiation. Non-irradiated (A) versus irradiated (B) planarians stained for Smed-GluR which is not affected by irradiation. Non-irradiated (C) versus irradiated (D) planarians stained for *cintillo* which is not affected by irradiation. Non-irradiated (E) versus irradiated (F) planarians stained for *smedwi-2* whose expression is lost after irradiation. Non-irradiated (G) versus irradiated (H) planarians stained for *sme-let-7b*; the expression of the miRNA is not affected by irradiation. Non-irradiated (I) versus irradiated (J) planarians stained for *sme-mir-31b*; the expression of the miRNA is not affected by irradiation. The next miRNAs are all affected by irradiation. Not irradiated (K) versus irradiated (L) planarians stained for *sme-mir-71b/2d/752*. Non-irradiated (M) versus irradiated (N) planarians stained for *sme-mir-7b*; note that in the irradiated some expression remains in the posterior midline (arrow) as well as around the pharynx. Non-irradiated (O) versus irradiated (P) planarians stained for *sme-mir-36*. Non-irradiated (Q) versus irradiated (R) planarians for *sme-mir-92*. Non-irradiated (S) versus irradiated (T) planarians for *sme-mir-124a*; note that in the irradiated animals a little of the staining remains in the posterior midline (arrow). Non-irradiated (U) versus irradiated (V) planarians for *sme-mir-124b*; note that in the irradiated a little of the staining remains in the posterior midline (arrow). Non-irradiated (W) versus irradiated (X) planarians for *sme-let-7c*; note that in the irradiated a little of the staining remains around the pharynx (arrow). Non-irradiated (Y) versus irradiated (Z) planarians for *sme-mir-2c/71c*. Non-irradiated (A') versus irradiated (B') planarians for *sme-mir-67*; note that in the irradiated a little of the staining remains in the posterior midline (arrow). (A-B, G-J) Ventral views; (C-F, K-B') dorsal views. Scale bars indicate 1 mm.

mir-7b, sme-mir-36, sme-mir-92, sme-mir-124a and sme-mir-124b expression are lost or greatly reduced after lethal doses of gamma radiation (Fig. 3K-V). Tissue specific markers of differentiated cell types like *Smed-GluR* (F. Cebrià and P. Newmark) or *cinillo* (Oviedo *et al.*, 2003) were not affected (Fig. 3A-D) whereas expression of *smedwi-2* (Reddien *et al.*, 2005a) was completely lost (Fig. 3E, F). For sme-mir-7c loss of parenchymal expression was difficult to interpret as in non-irradiated adults parenchymal expression is faint. The same is true for two other miRNAs, sme-mir-C and sme-mir-747, where expression in the parenchyma of non-irradiated intact animals is also weak.

We also examined some miRNAs which, although they didn't have the typical expression pattern corresponding to neoblast markers known to date, are expressed in the parenchyma. Most of these, for instance sme-let-7b (Fig. 3G, H), sme-lin-4a, sme-mir-1a, sme-mir-2a-1/71a1, sme-mir-2a-2/71a2 and sme-mir-745 (data not shown), were not affected by irradiation. In contrast, sme-let-7c and sme-mir-2c were no longer expressed after irradiation (Fig. 3W-Z).

For the miRNAs with expression in the germline of sexual planarians (see above) we found that sme-mir-36 and sme-mir-67 (Fig. 3O-P, A'-B') showed no expression after irradiation in asexual planarians but the expression of sme-lin-4c remained (data not shown). This suggests a correlation between sme-mir-36 and sme-mir-67 expression in neoblasts and germ cells, which is true of other neoblast/germ line markers (Shibata *et al.*, 1999). In the case of sme-mir-756 we observed no expression in the asexual strain suggesting that it might only be expressed at significant levels in the sexual strain.

As a control, we performed ISH on irradiated animals for some of the miRNAs that clearly seemed to be expressed in differentiated cells, for instance sme-mir-31b (Fig. 3I, J), sme-mir-87b or sme-mir-749 (data not shown) that were not affected by irradiation.

Whole-mount in situ hybridization with LNA probes confirms miRNA expression patterns obtained with nascent transcript probes

In order to confirm that the miRNA expression patterns we observed using probes to nascent transcripts were indicative of mature miRNA expression we performed whole-mount ISH with LNA probes for two miRNAs, sme-let-7c and sme-mir-67. ISH with LNA probes was successful showing that future work on miRNAs of particular interest can be done with this more costly approach.

We observed the same expression pattern with LNA probes as we observed for our unprocessed transcript probes for sme-let-7c

and sme-mir-67 in asexual organisms (Supplementary Fig. 6). Furthermore, we can say, at least for sme-let-7c and sme-mir-67 that these miRNAs are probably not being post-transcriptionally regulated, at least spatially, in planarians.

Whole-mount fluorescent in situ hybridization with nascent transcript probes show sub-cellular localization to the nucleus

Sub-cellular localization of typical mRNA transcripts has not been previously described in planarians. In order to confirm that the miRNA expression patterns with nascent probes are detecting primary miRNA transcripts in the nucleus we performed confocal imaging on whole-mount fluorescent ISH for several miRNA nascent probes using one LNA probe and two protein coding genes as controls.

Localization of *smedwi-2* (Reddien *et al.*, 2005a) and *cinillo* (Oviedo *et al.*, 2003) transcripts was, as expected, predominantly in the cytoplasm (Fig. 4A-H). However, increasing the strength of excitation allowed the faint detection of nuclear dots in addition to cytoplasmic expression (Fig. 4B, arrows in the high magnification panel). In addition, localization of *smedwi-2* appeared to be peri-nuclear. In contrast, for all the miRNA nascent probes analysed, sme-mir-749, sme-mir-87b, sme-mir-31b and sme-let-7b the transcript was detected exclusively in the nucleus (Fig. 4I-X). Expression was never observed in the cytoplasm. Cells with a low intensity of fluorescence show 2 dots whereas cells with higher fluorescence stain more broadly in the nucleus (Fig. 4P asterisk). Confocal sections show that sme-let-7c mature transcripts are detected strongly in the dorsal midline and only weakly at the ventral one (Fig. 4Y and Supplementary Fig. 7). We would expect to have a similar dorso-ventral distribution for the other miRNAs with similar midline expression. Finally sme-let-7c LNA probe exclusively detected the mature miRNAs in the cytoplasm (Fig. 4Z-E').

Demonstration of sub-cellular localization confirms the specificity of miRNA nascent probes for the detection of primary miRNAs in the nuclei. It also suggests that further studies of sub-cellular transcript localisation maybe important for identifying those genes expressed in peri-nuclear nuage or chromatoid bodies in planarians.

Discussion

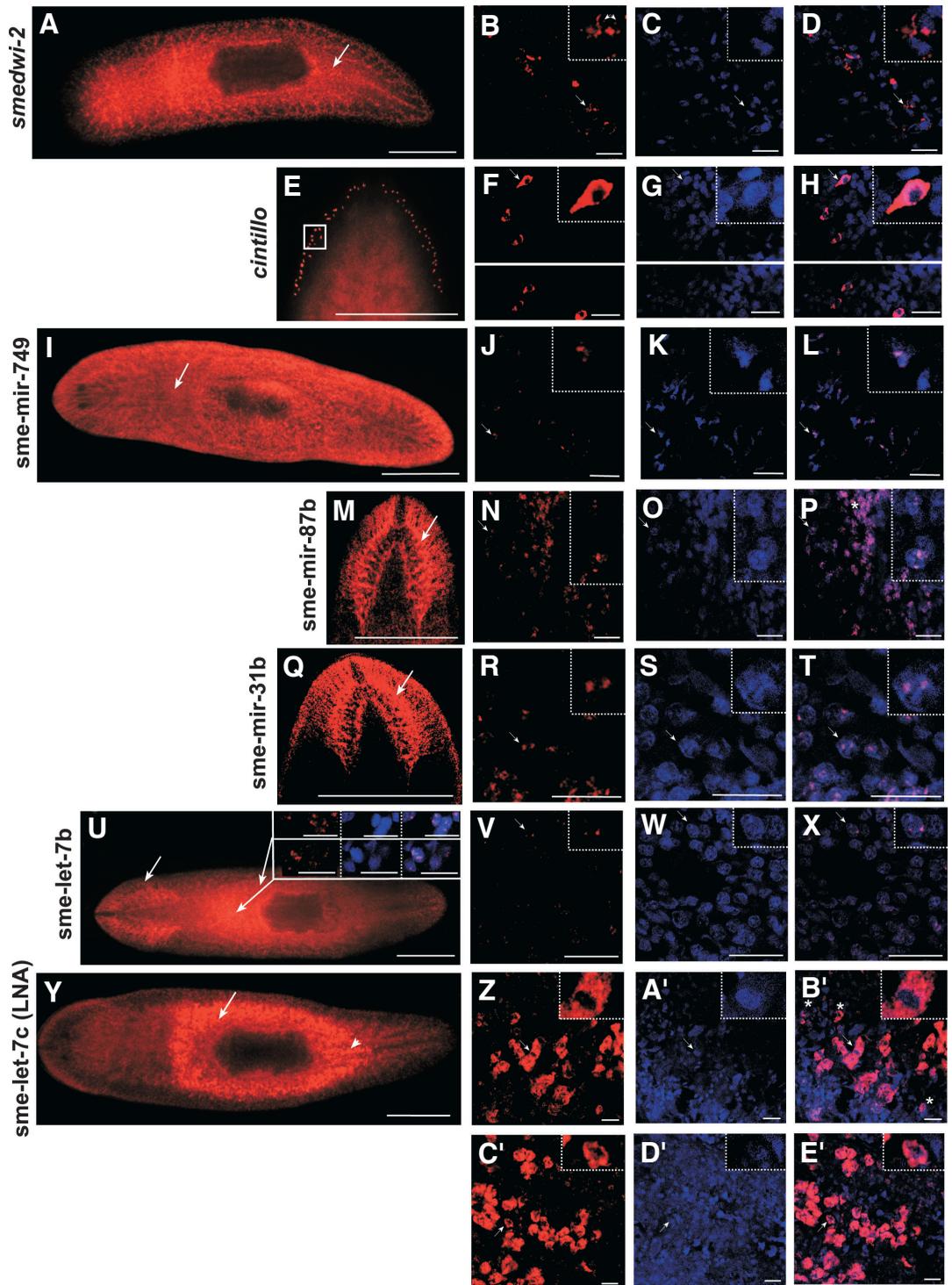
We have shown that detection of miRNAs in planarians can be achieved by ISH detection using probes to primary miRNA transcripts as previously reported for *Drosophila* embryos (Aboobaker *et al.*, 2005). We have been able to characterize 41 miRNA expression patterns in the asexual strain and one miRNA expres-

(Legend continued from opposite page)

planarian; the arrows indicate the areas which are analyzed by confocal microscopy in the upper right panels of U and in V-X. The upper right panels in U show single confocal section of a labelling to detect sme-let-7b nascent transcript in red, the nuclei counterstained with Hoechst in blue and the merged image where sme-let-7b labelling can be observed as two dots. (V-X) Single confocal section of the area indicated by an arrow in U showing a labelling to detect sme-let-7b nascent transcript in red (V) and the nuclei counterstained with Hoechst (W); the merged image is seen in X where sme-let-7b labelling can be observed as two dots. (Y) Dorsal view of a whole-mount fluorescent ISH for sme-let-7c using the LNA probe on an intact planarian; the arrow indicates the ventral area which is analyzed by confocal microscopy in Z-B' whereas the head arrow indicates the dorsal midline which is analysed in C'-E'. (Z-E') Single confocal section of a labelling to detect sme-let-7c mature miRNA by using a LNA probe in red (Z and C') and the nuclei counterstained with Hoechst (A' and D'); the merged image are seen in B' and E' where sme-let-7c mature miRNA detected by an LNA probe can be observed exclusively at the cytoplasm; note that in Z-B' at least two different cell types are stained: big cells, one of them (arrow) shown in the high magnification panel and small cells (asterisks). From the whole-mount images, A, E, I, U and Y have been taken with a fluorescence stereomicroscope whereas M and Q are confocal projections. The small squares in the upper right corner of all the confocal slices show a high magnification view of the cell indicated by an arrow. Scale bars in all the whole-mount images (A, E, I, M, Q, U and Y) indicate 1 mm. Scale bars in all the confocal sections indicate 20 μ m.

Fig. 4. Sub-cellular localization of mRNAs, miRNA nascent transcripts and miRNA mature transcripts.

This figure illustrates that mRNAs of typical genes predominantly localize sub-cellularly to the cytoplasm; nascent miRNA transcripts exclusively localize sub-cellularly to the nucleus, while mature miRNA transcripts exclusively localize sub-cellularly to the cytoplasm. (A) Dorsal view of a whole-mount fluorescent ISH for *smedwi-2* on an intact planarian; the arrow indicates the dorsal midline where it is analyzed by confocal microscopy in B-D. (B-D) Single confocal section of a labelling to detect *smedwi-2* mRNA in red (B) and the nuclei counterstained with Hoechst (C); the merged image is seen in D where *smedwi-2* labelling can be observed predominantly around the nucleus in the cytoplasm. However, with increased laser intensity, the staining can also be observed in the nucleus as two dots (B) (high magnification panel, double head arrows). (E) Dorsal view of a whole-mount fluorescent ISH for *cintillo* on an intact planarian; the image shows only the head of the intact planarian; the square indicates the group of cells which are analyzed by confocal microscopy in F-H. (F-H) Single confocal section of a labelling to detect *cintillo* mRNA in red (F) and the nuclei counterstained with Hoechst (G); the merged image is seen in H where *cintillo* labelling can be observed predominantly at the cytoplasm; F, G and H are divided in two images, because they correspond to single confocal sections from different focal planes of the same sample. (I) Ventral view of a whole-mount fluorescent ISH for *sme-mir-749* on an intact planarian; the arrow indicates the area which is analyzed by confocal microscopy in J-L. (J-L) Single confocal section of a labelling to detect *sme-mir-749* nascent transcript in red (J) and the nuclei counterstained with Hoechst (K); the merged image is seen in epidermal cells. (M) Ventral view of a whole-mount fluorescent ISH for *sme-mir-87b* on an intact planarian; the image shows only the head of the intact planarian; the arrow indicates the area which is analyzed by confocal microscopy in N-P. (N-P) Single confocal section of a labelling to detect *sme-mir-87b* nascent transcript in red (N) and the nuclei counterstained with Hoechst (O); the merged image is seen in P where *sme-mir-87b* labelling can be observed as two dots; an area with higher laser confocal intensity shows that staining is still observed only in the nucleus (P, asterisk). (Q) Ventral view of a whole-mount fluorescent ISH for *sme-mir-31b* on an intact planarian; the image shows only the head of the intact planarian; the arrow indicates the area which is analyzed by confocal microscopy in R-T. (R-T) Single confocal section of a labelling to detect *sme-mir-31b* nascent transcript in red (R) and the nuclei counterstained with Hoechst (S); the merged image is seen in T where *sme-mir-31b* labelling can be observed as two dots. (U) Ventral view of a whole-mount fluorescent ISH for *sme-let-7b* on an intact



(U) Ventral view of a whole-mount fluorescent ISH for *sme-let-7b* on an intact

by confocal microscopy in J-L. (J-L) Single confocal section of a labelling to detect *sme-mir-749* nascent transcript in red (J) and the nuclei counterstained with Hoechst (K); the merged image is seen in epidermal cells. (M) Ventral view of a whole-mount fluorescent ISH for *sme-mir-87b* on an intact planarian; the image shows only the head of the intact planarian; the arrow indicates the area which is analyzed by confocal microscopy in N-P. (N-P) Single confocal section of a labelling to detect *sme-mir-87b* nascent transcript in red (N) and the nuclei counterstained with Hoechst (O); the merged image is seen in P where *sme-mir-87b* labelling can be observed as two dots; an area with higher laser confocal intensity shows that staining is still observed only in the nucleus (P, asterisk). (Q) Ventral view of a whole-mount fluorescent ISH for *sme-mir-31b* on an intact planarian; the image shows only the head of the intact planarian; the arrow indicates the area which is analyzed by confocal microscopy in R-T. (R-T) Single confocal section of a labelling to detect *sme-mir-31b* nascent transcript in red (R) and the nuclei counterstained with Hoechst (S); the merged image is seen in T where *sme-mir-31b* labelling can be observed as two dots. (U) Ventral view of a whole-mount fluorescent ISH for *sme-let-7b* on an intact

sion pattern in the sexual that we did not detect in the asexual strain. Some miRNAs are implicated by their expression patterns to be potential stem cell regulators and even germ stem cell regulators. This is the first Lophotrochozoan animal where miRNA expression patterns have been determined, albeit post-embryonically. This is also the first time that sub-cellular localization of transcripts has been demonstrated in the planarian model system. We observe a broad set of miRNA expression patterns, many of them with conserved patterns with respect to their orthologs in other animals. These data provide an important starting point for the functional analysis of these miRNAs in planarians.

We can not rule out a technical basis for failure to detect the remaining miRNAs. However, it seems more likely they are being transcribed at low levels, are expressed during very precise regeneration stages we have not covered or are expressed only during starvation or other specific life history scenarios we did not test. Alternatively, the primary miRNA transcripts may be too short or processed too rapidly to be detected by this method.

It has been shown in the past 5 years that many vertebrate miRNAs are enriched in the nervous system, which is consistent with the complexity of neural cell identities and the necessity for neural cells to have a perfect spatio-temporal control of mRNA translation for assuring their synaptic development and plasticity (Kim *et al.*, 2004; Sempere *et al.*, 2004). It is now becoming increasingly evident that miRNAs are essential for synaptic development and that their misregulation might contribute to the etiology of neuronal diseases characterized by synaptic dysfunction (Fiore and Schratt, 2007; Schratt *et al.*, 2006). An advantage of whole-mount ISH compared to Northern blot analysis is that not only temporal but also spatial patterns can be assessed. We have managed to show 36 miRNA loci patterns. Of these more than 70% have expression in the nervous system of intact asexual planarians. The expression of many of these miRNAs corresponds to a region suggested to be involved in signal transduction to the VNC (Nakazawa *et al.*, 2003) suggesting a direct link between miRNAs with synaptic development. Although the planarian brain has long been considered as one of the “simplest” brains within the Bilateria (Keenan *et al.*, 1981), it is now becoming evident that the planarian CNS and in particular the brain actually shows a complex cytoarchitecture that is functionally regionalized allowing the performance of complex processes (Cebrià *et al.*, 2002; Nakazawa *et al.*, 2003; Cebrià *et al.*, 2007). A brain capable of complex responses to the environment within the context of an organism that is continuously remodeling its body (Morgan, 1898) including the CNS (Baguña and Romero, 1981) must require a very precise regulation of synaptic development and plasticity. miRNAs may play an important role in this process, and the variety of miRNAs expressed in and in close proximity to the CNS supports this.

Emerging evidence suggests that miRNAs play an essential role in stem cell self-renewal and differentiation (Yang *et al.*, 2001; Houbaviv *et al.*, 2003; Kuwabara *et al.*, 2004; Suh *et al.*, 2004; Cheng *et al.*, 2005; Forstmann *et al.*, 2005; Hatfield *et al.*, 2005; Kanellopoulou *et al.*, 2005; Lee *et al.*, 2005; Murchison *et al.*, 2005; Tang *et al.*, 2006; Yu *et al.*, 2007). Almost 80% of the miRNAs are expressed in the planarian parenchyma. Of

these we found that *sme-mir-752/2d/71b*, *sme-mir-7b*, *sme-mir-36*, *sme-mir-92*, *sme-mir-124a* and *sme-mir-124b*, which are miRNAs with a typical neoblast-like pattern, are expressed in neoblasts/irradiation sensitive cells. *sme-mir-7c* has the same characteristic pattern indicative of neoblast expression, thus is very likely to be expressed in neoblasts although we could not confirm this unambiguously with our irradiation experiments.

We have found a group of miRNAs that appear to be expressed in the germline associated tissues in planarians. Of these *sme-mir-36* and *sme-mir-67* are also expressed in neoblasts/irradiation sensitive cells in asexual planarians. They have expression patterns that are the same as previously described markers of germ stem cells (Handberg-Thorsager and Saló, 2007) but different to markers of differentiated germ cells (Zayas *et al.*, 2005). This suggests that the *sme-mir-36* and *sme-mir-67* miRNAs are expressed in germ stem cells. As yet little is known about the function of these conserved miRNAs in other systems. The finding that *sme-lin-4c* expression is not affected by irradiation suggests that it is not expressed in neoblasts and that its expression in germline tissues may be restricted to differentiated ova rather than germ stem cells. This agrees with its expression pattern centrally in the lumen of the ovary, compared to the more lateral/peripheral expression of the other miRNAs.

The rest of the miRNAs expressed in the parenchyma show slightly different parenchyma patterns from the “typical neoblast” pattern (Reddien *et al.*, 2005a). We found that *sme-let-7c*, *sme-mir-2c/71c* and *sme-mir-67* are expressed in neoblasts/irradiation sensitive cells. But *sme-mir-2a1/71a1*, *sme-mir-2a2/71a2*, *sme-mir-281* and *sme-mir-745* that have very similar expression patterns were not affected by irradiation. For simplification we describe this pattern as “medial parenchyma, partial posterior midline, brain ganglia” (Table 1). However, we note that the irradiation insensitive expression patterns in this group (*sme-mir-2a1/71a1*, *sme-mir-2a2/71a2*, *sme-mir-281* and *sme-mir-745*) all have expression in the posterior midline just behind the pharynx (Area A in blue in Supplementary Fig. 8). For those miRNA expression patterns in this group that were irradiation sensitive, the expression in the posterior midline is further posterior to area A (area B in dark brown in Supplementary Fig. 8). This suggests that the cells in area A (irradiation insensitive) and area B (irradiation sensitive) are distinct midline populations. In agreement with the existence of these cell populations, miRNAs with posterior midline expression immediately proximal to the pharynx (in area A) are not affected by irradiation and some miRNA expression patterns (*sme-mir-7b*, *sme-mir-124a*, *sm-mir-124b* and *sme-mir-67*) maintain expression in this subset of cells after irradiation, while expression is lost elsewhere (Supplementary Fig. 8). Other previously described genes show a similar pattern of expression in area A (as well as elsewhere). For instance, *smedinx-1* is expressed in this area (Oviedo and Levin, 2007) and its expression is not affected by irradiation. The marker TCEN49, expressed in most cell types except epidermal cells (Bueno *et al.*, 1996; Vispo *et al.*, 1996) and a Zinc-dependent metalloprotease (Sánchez Alvarado *et al.*, 2002) are also expressed in the posterior midline just posterior to the pharynx. More studies need to be done to investigate the nature of these cells, what is clear is that they express a complex set of miRNAs.

An interesting observation is that there are diverse restricted expression patterns along the anterior-posterior (AP) axis in the

parenchyma and nervous system that suggest a function for miRNAs in modulating or maintaining the AP polarity during planarian homeostasis. Examples include the posteriorly enhanced expression of *sme-mir-124c* (Fig. 1Z) or *sme-mir-31b* (Fig. 1U), *sme-mir-Ea* which is expressed in the posterior regions of the digestive system (Fig. 1C) and *sme-mir-C* (Fig. 1I) and *sme-mir-1a* (Supplementary Fig. 1B) which are expressed only in the mid-posterior excretory system.

An important caveat to our results is that nascent transcription may not precisely parallel the accumulation of mature miRNAs. While using LNA oligonucleotide probes would avoid this (Wienholds *et al.*, 2005), as well as have lower background levels and larger number of target molecules for detection, the big disadvantage is that it is a technique not affordable for most labs. At least for *sme-let-7c* and *sme-mir-67* there is a parallel accumulation of nascent transcripts and mature miRNAs and we believe this to be the case for most, but not all, miRNAs.

Our data gives the first expression patterns and the first insight into miRNA function in a Lophotrochozoan, the planarian *Schmidtea mediterranea*. Additionally, we now have an idea which miRNAs are expressed in the planarian stem cell population and may therefore be involved in self-renewal and differentiation. Once potential targets are identified by bioinformatic means, a greater insight into the role of miRNAs in various aspects of planarian biology will be possible. The functional study of miRNAs awaits the development of a reverse genetic technique capable of removing the expression of individual miRNAs. While morpholinos have been shown to work in other systems the multicellular nature of the planarian system prevents this approach in adult planarians. RNAi approaches can be used to investigate predicted miRNA targets that can be experimentally validated in other misexpression systems (Reddien *et al.*, 2005b). Ultimately improvements in the currently limited transgenic approaches will allow miRNAs to be over-expressed and possibly their functions elucidated more directly (González-Estévez *et al.*, 2003). The global impact and role of miRNAs may be assessed by studying the different components of the miRNA biogenesis pathway. Once this is done we will be able to better understand the role of miRNAs in regulating planarian homeostasis and regeneration and the roles of miRNAs in self-renewal and differentiation.

Materials and Methods

Animals

Planarians used in this work belong to the species *Schmidtea mediterranea* asexual strain originally provided by Dr. Emili Saló (University of Barcelona, Barcelona, Spain) and were collected in a fountain in Montjuïc, Barcelona, Spain. The sexual strain was collected in Sardinia, Italy. Animals were maintained at 19°C, cultured in Trent (UK) river water and fed once per week with organic chicken liver. Animals were starved for one week prior to experiments.

miRNAs nascent templates

Genomic DNA extraction was done as described previously for nematodes (Aboobaker and Blaxter, 2003). BLAST searches were performed on an assembly of the *S. mediterranea* genome (<http://genome.wustl.edu>) to search for the genomic region surrounding the mature planarian miRNAs (miR base, <http://microrna.sanger.ac.uk/>). We generated 400bp-700bp templates (Supplementary table) with T7 promoters appended to the antisense strand, from all known planarian miRNA loci (Palakodeti *et al.*, 2006). In most cases, the pre-miRNA hairpin resides in the center of

this probe. Where multiple miRNAs reside in a small cluster, we designed one or multiple probes that span the cluster.

LNA probes

Two miRCURY™ 5'-DIG LNA detection probes labelled were obtained from Exiqon (Vedbæk, Denmark): *sme-let-7c* (5'-aaccttttgagtcactactca-3') and *sme-miR-67* (5'-acccctgtcatggaggttgga-3').

Whole-mount in situ hybridization experiments

Digoxigenin-labelled RNA probes for the nascent templates were prepared by using an *in vitro* labelling kit (Roche, Basel, Switzerland). Whole mount ISH hybridization was carried out as described previously (Umesono *et al.*, 1999) with the following modifications. Proteinase K treatment (20 µg/ml) was performed for 8 min on the asexual strain and for 12 min on the sexual strain. A triethanolamine treatment was performed as described by Nogi and Levin (2005). All the nascent probes were used to a concentration of 1ng/µl and the hybridizations were carried out at 55 °C for 17 hours. *sme-let-7c* LNA probe was used at a concentration of 1nM and the hybridizations were carried out at 54 °C for 17 hours. *sme-miR-67* LNA probe was used at a concentration of 15nM and the hybridizations were performed at 57 °C for 17 hours. All washes, for both nascent and LNA probes were performed at the corresponding hybridization temperature and as follows: 100% Hybridization solution for 15 min, 75% Hybridization solution / 25% (2xSSC; 0.1% Triton X-100) for 10 min; 50% Hybridization solution / 50% (2xSSC; 0.1% Triton X-100) for 10 min; 25% Hybridization solution / 75% (2xSSC; 0.1% Triton X-100) for 10 min; two washes of (0.2xSSC; 0.1% Triton X-100) for 30 min each; two washes of (0.2xSSC; 0.1% Triton X-100) for 30 min each. For LNA probes, Tween-20 was used instead of Triton X-100 for the washes, in Buffer I and in Buffer II.

The developing time for some miRNAs was very short (up to 3 hours); however, some miRNAs required much more time (up to 8 hours) to develop, consequently resulting in more background. In the case of the two LNA probes the developing time was always very short compared to the corresponding nascent probe.

Samples were observed through a Zeiss Discovery V8 from Carl Zeiss and the images were taken with an AxioCam MRC from Carl Zeiss.

Positive controls (data not shown) for all ISH were performed with *smed_noggin1* (Molina *et al.*, 2007) and *smedwi-2* (Reddien *et al.*, 2005a).

Whole-mount fluorescent in situ hybridization experiments

Whole-mount fluorescent ISH hybridization was carried out as described previously (Cebrià *et al.*, 2007) with the following modifications. Samples were developed using Tyramide Signal Amplification (Perkin Elmer) at a concentration of 1:50 in 1x Amplification Buffer for 20 minutes. Nuclei were stained with 0.5 µg/ml Hoechst 33342 overnight at 4 °C

We observed that this protocol is perfectly adapted for those miRNAs whose expression usually develops in up to 3 hours using NBT/BCIP but it does not work for the rest.

Nascent and LNA probes were used at the same concentration as for the conventional ISH. The genes *cintillo* (Oviedo *et al.*, 2003) and *smedwi-2* (Reddien *et al.*, 2005a) were used as controls.

Samples were mounted in SlowFade Antifade (Molecular Probes) to be analyzed in a Leica MZ16F fluorescence stereomicroscope and recorded on a Leica DFC 300Fx camera (Leica Lasertechnik, Heidelberg). Confocal laser scanning microscopy was performed with a Leica SP2 confocal laser scanning microscope (CLSM) (Leica Lasertechnik, Heidelberg).

Irradiation

Intact asexual and sexual planarians were irradiated at 96 Gy (6 Gy/

min for 16 min) of gamma radiation using a sealed ^{137}Cs source (Gammacell-1000 Elite, Nordion, Kanata, Canada). Fixation for whole-mount ISH was performed at 6 days post-irradiation.

Acknowledgments

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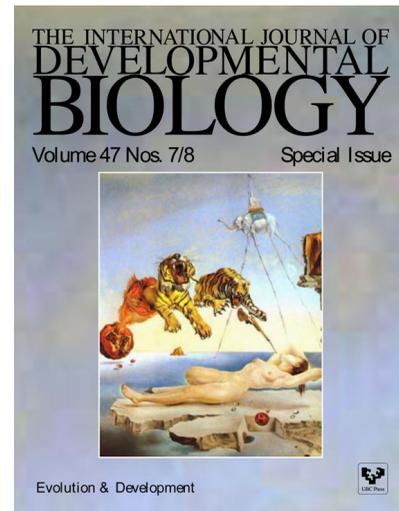
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