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Vasa and the germ line lineage in a colonial urochordate

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ABSTRACT

Germ cell sequestering in Animalia is enlightened by either, launching true germ line along epigenetic or preformistic modes of development, or by somatic embryogenesis, where no true germ line is set aside. The research on germ line-somatic tissue segregation is of special relevancy to colonial organisms like botryllid ascidians that reconstruct, on a weekly basis, completely new sets of male and female gonads in newly formed somatic tissues. By sequencing and evaluating expression patterns of BS-Vasa, the *Botryllus schlosseri* orthologue of Vasa, in sexually mature and asexual colonies during blastogenesis, we have demonstrated that the BS-Vasa mRNA and protein are not expressed exclusively in germ cell lineages, but appeared in cells repeatedly emerging *de novo* in the colony, independently of its sexual state. In addition, we recorded an immediate Vasa response to cellular stress (UV irradiation) indicating additional functions to its germ line assignments. To confirm germ lineage exclusivity, we examined the expression of three more stem cell markers (BS-Pl10, Bl-piwi and Oct4). Vasa co-expression with Pl10 and Oct4 was detected in germ line derivatives and with Bl-piwi in somatic tissues. Presumptive primordial germ cells (PGC-like cells), that are $Vasa^{+}/Pl10^{+}/Oct4^{+}$ and 6–12 μm in diameter, were first detected in wrapped-tail embryos, in oozooids, in sexual/asexual colonies, within a newly identified PGC niche termed as 'budlet niche', and in circulating blood borne cells, indicating epigenetic embryogenesis. Alternatively, BS-Vasa co-expression with piwi orthologue, an omnipresent *bona fide* stemness flag, in non germ line cell populations, may indicate germ cell neogenesis (somatic embryogenesis) in *B. schlosseri*. Both alternatives are not necessarily mutually exclusive.

Introduction

The competence to demarcate between the immortal totipotent germ line (considered also the 'stem cells of the species'; Wylie, 1999, 2000) and the mortal soma is restricted to the kingdom Animalia. Germ line sequestering is manifested by setting apart a small population of primordial germ cells from other cell lineages through a sex-specific differentiation program called gametogenesis, a phenomenon also regulated by signals from the environment. A shorter ontogenic window for germ line sequestering further limits the opportunity for introducing somatic variants into the germ line (Buss, 1983). Evaluating time and mode of germ line sequestering is important for phylogenetic considerations and understanding of developmental aspects, such as maintaining germ cells throughout ontogeny (Blackstone and Jasker, 2003; Extavour and Akam, 2003).

The literature recognizes several modes of germ cell sequestering in organisms. Using similar terminology, at least, two distinctive approaches are used to categorize the origin of the germ line. One approach (Extavour and Akam, 2003) classifies two modes of germ cell specification, either at the beginning of embryogenesis when inheriting maternally cytoplasmic determinants (preformistic mode of development), or at later ontogenic stages through inductive signals from neighboring tissues (epigenetic mode of development). In other development texts, the terms 'epigenetic' and 'preformation' were synonyms to 'regulative' and 'mosaic', respectively (Buss, 1983). Above partly metaphysical terms, are based on the 18th century philosophical debates seeking to describe the development of individual organic form. Epigenesis (addressing the creative dimensions of embryogenesis), as opposed to preformation (in which, traditionally, the egg or the germ line, supposedly houses, from the "beginning" the homunculus, a tiny version of the adult) refers therefore, in current germ line usages, to other meanings (i.e., Extavour and Akam, 2003). The second approach (Buss 1982, 1983; Blackstone and Jasker, 2003) labels germ cell sequestering by three modes of ontogenetic developments. The first is the mode of somatic embryogenesis, assigned to those organisms that, from birth to death, are capable of developing germ cells from certain tissues at any ontogenic phase. The second mode, epigenetic development, is characteristic to organisms in which, at least, some potentially totipotent cell lines remain undetermined until relatively late in ontogeny. The third mode, preformistic development, tags organisms whose entire repertoire of germ cell lineages is irreversibly determined early in ontogeny. According to the latter classification, many animals, including placozoans, sponges, cnidarians, platyhelminths, nemerteans, entoproctans, ectoproctans, annelids, hemichordates and urochordates are capable of somatic embryogenesis (Buss 1982, 1983;

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Blackstone and Jasker, 2003), a mode of development that was ignored in Extavour and Akam's (2003) classification. This is in addition to plants, fungi, and multicellular protists that ubiquitously exhibit somatic embryogenesis.

The above ambiguity, which had probably seeped into literature from Weismann's first demarcation between germ and soma (Weismann, 1892), could project on our understanding of the cellular and molecular portraits underlying the segregation of germ cells from somatic cells in conjunction with other developmental processes. An example is Buss's finding (1983) that preformistic mode of development (according to Buss's definition) is absent in all groups capable of asexual reproduction, namely, the formation of more than one ramet per genet. Therefore, at least some of these organisms lack a true germ line that clearly diverges from somatic tissues (somatic embryogenesis mode of development). Above discussion is relevant to colonial organisms, like botryllid ascidians. This group of colonial urochordates presents a unique case of somatic plasticity, a model system for development and germ cell sequestering (Stoner et al., 1999; Laird et al., 2005; Rinkevich et al., 2007; Ballarin, 2008; Manni et al., 2008).

The ascidian *Botryllus schlosseri* is composed of a few to thousands of genetically identical modules (zooids), embedded within a gelatinous matrix, the tunic. The soma in this group of organisms is hierarchically arranged in three successive generations of modules, the mature feeding zooids and two generations of buds, the primary buds that are attached to the zooids and the secondary buds (budlets), emerging from the primary buds. A colony grows through a highly coordinated weekly development process called blastogenesis (Berrill, 1950, 1951; Mukai and Watanabe, 1976b; Mukai, 1977). Once a week, zooidal tissues die through a massive apoptotic process (Lauzon et al., 1992, 1993, 1996; Cima et al., 2003; Ballarin et al., 2008), concomitantly replaced by mature primary buds. The free-swimming tadpole larva metamorphoses into a sessile singular module (called oozooid), which multiplies through successive blastogenic cycles. *B. schlosseri* is a hermaphroditic species with male and female gonads situated side by side in each zooid. Ova mature through several blastogenic cycles and migrate to the developing buds of the succeeding generations (Sabbadin et al., 1992) until ovulation and fertilization are finally attained with precise coordination between sexual reproduction and blastogenesis (Berrill, 1950; Milkman, 1967; Mukai and Watanabe, 1976a). Embryogenesis follows the blastogenic cycle, so that embryos mature and hatch just prior to the regression of adult zooids.

It is commonly accepted that the Vasa gene products are the best molecular markers for identification of the germ line lineage in many organisms (reviewed in Raz, 2000; Linder and Lasko, 2006). As in other organisms, Vasa mRNA and protein were also found to flag the germ line derivatives in various botryllid ascidians and other budding tunicates (Sunanaga et al., 2006, 2007; Brown and Swalla, 2007). Vasa is a member of a large DEAD Box RNA helicases family of proteins known to function in wide aspects of RNA metabolism, including transcription, splicing, RNA export, ribosome biogenesis, RNA degradation, translation initiation and gene expression in organelles (Linder, 2006). This family of proteins is characterized by nine conserved motifs; among them is the DEAD motif (Asp-Glu-Ala-Asp), which gives the name to the entire family. This family is composed of several subfamilies according to their sequences and functions (Abdelhaleem, 2005; Cordin et al., 2006; Fuller-Pace, 2006; Linder, 2006). Vasa and Pl10 are two such subfamilies (Rosner and Rinkevich, 2007).

By isolating and observing expression patterns of BS-Vasa, the *B. schlosseri* orthologue of Vasa, we demonstrated in this study that the Vasa mRNA and protein not only are expressed in germ line derivatives, but are also expressed in non-germ line lineages, like the test cells and are also elevated by UV radiation stress. In order to confirm the germ line identity we studied expression patterns of additional stem cell markers. These included BS-Pl10 (a *B. schlosseri* Pl10 orthologue), which is also expressed at early staged ascidians germ cells (Rosner et al., 2006); Oct4, one of the most reliable markers for stem cells and primordial germ cells (Donovan, 2001; Cheng et al., 2007); and Bl-piwi, a member of the piwi family, known for essential functions during spermatogenesis (Kuramochi-Miyagawa et al., 2004), germ line regulation (Carmell et al., 2007; O'Donnell and Boeke, 2007) and stem cell regeneration and homeostasis (Palakodeti et al., 2008). Cumulatively, the results, suggest an epigenetic mode of development. Additionally, based on BS-Vasa mode of expression, either parallel somatic embryogenesis or non-germ line roles of Vasa in botryllid ascidians biology are proposed.

Materials and methods

Animals

B. schlosseri colonies (Satoh, 1994; Fig. 1A) collected at Monterey, Half Moon Bay and Moss Landing Marinas, California, have been reared at 20 °C as described by Rinkevich and Shapira (1998).

Isolation of BS-Vasa cDNA

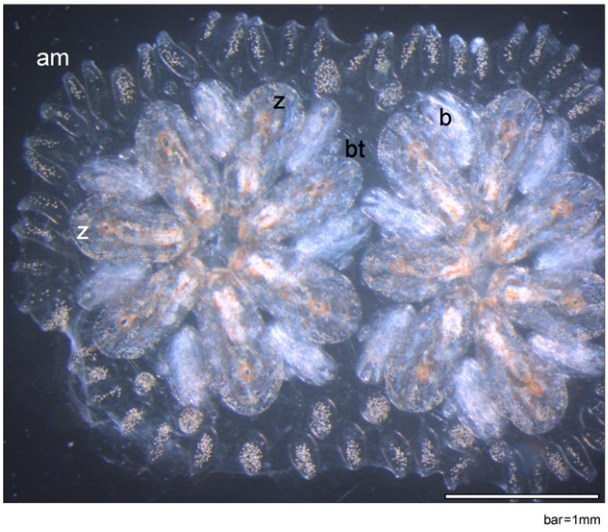
We used designed (Fujimura and Takamura, 2000) degenerate primers (synthesized by IDT-Integrated DNA Technologies, IOWA, USA) to isolate an initial 378 bp fragment from BS-Vasa conserved region. Regions 5′ and the 3′ of this fragment were isolated using 'SMART RACE cDNA Amplification Kit' (Clontech, CA, USA).

Antibodies

Specific polyclonal anti BS-Vasa protein were elicited against the polypeptide GAVGTHHGPKGGRFGARDC, a sequence highly specific within BS-Vasa protein, which does not exist in BS-Pl10. The polypeptide was coupled with chicken albumin and used to immunize two rabbits (Hy-labs Biotechnologies, Rehovot, Israel). Bl-piwi polyclonals, similarly elicited against the *Botrylloides leachi*

Fig. 1. *Botryllus schlosseri*. (A) A typical colony with two colonial systems and 14 zooids at blastogenic stage C, harboring well developed primary buds and extended vasculature system. Abbreviations: am—ampulla; b—primary bud; bt—budlet; z—zooid. (B) Phylogenic analysis of the conserved region (about 380 amino acid) of the Vasa (DDX4) and the DDX3 (Pl10) subfamilies of the DEAD Box family. The analyzed sequences include protein fragments from *B. schlosseri* BS-Vasa and *B. schlosseri* BS-PL10, and protein fragments from the following orthologues: DDX3 of *Canis familiaris* accession no: XP_861449.1; DDX3 of *Danio rerio* accession no: CAA73349.1; DDX3 of *Homo sapiens* accession no: BAD92220.1; DDX3 of *Macaca mulatta* accession no: XP_001095294.1; DDX3 of *Rattus norvegicus* accession no: BC085914; DDX3X of *Mus musculus* accession no: NM_010028.3; PL10 (D1Pas1) of *M. musculus* accession no: NM_033077.2; DDX3Y of *Pan troglodytes* accession no: NP_001008986.1; DDX3Y of *H. sapiens* accession no: NM_004660.3; PL10 of *Rana lessonae* accession no: AJ850054.1; PL10 of *Xenopus laevis* accession no: NM_001086814.1; DDX3Y of *Pongo abelii* accession no: CR857318.1; DDX3 of *Gallus gallus* accession no: NM_001030800.1; DDX3Y of *M. musculus* NM_001008986.1; PL10b of *Platynereis dumerilii* accession no: AM048814.1; PL10a of *P. dumerilii* accession no: AM048813.1; CnPL10 of *Hydra magnipapillata* accession no: AB047381.1; Belle of *Drosophila melanogaster* accession no: NM_080522.2; DED1 of *Saccharomyces cerevisiae* accession no: A6ZP47; DjVLGA of *Dugesia japonica* accession no: BAA34993; PoPL10 of *Ephydatia fluviatilis* accession no: BAB13309.1; DjVLGB of *D. japonica* accession no: BAA34994.1;Vasa of *Botrylloides violaceus* accession no: ABM74410.1; Vasa of *Botryllus primigenus* accession no: BAE44472.1; Vasa of *Polyandrocarpa misakiensis* accession no: BAE94497.1; Vasa of *Ciona savignyi* accession no: BAB12216.1; Vasa of *Ciona intestinalis* accession no: BAE93311.1; Vasa of *Leucopsarion petersii* accession no: BAD04052.1; Vasa of *D. rerio* accession no: AF461759_1; Vasa of *Monopterus albus* accession no: ABA54551.1; Vasa of *P. dumerilii* accession no: CAJ15139.1; Vasa of *Bos taurus* accession no: NP_001007820.1; Vasa of *M. mulatta* accession no: XP_001099856.1; Vasa of *H. sapiens* accession no: CAB70750.1; Vasa of *M. musculus* accession no: NP_034159.1; Vasa of *R. norvegicus* accession no: NP_001071115.1; Vasa of *R. lessonae* accession no: Q3MSQ8.1; Vasa of *G. gallus* accession no: NP_990039.1; CnVAS2 of *H. magnipapillata* accession no: BAB13308.1; CnVAS1 of *H. magnipapillata* accession no: BAB13307.1; PoVAS1 of *E. fluviatilis* accession no: BAB13310.1.

A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DED1 Saccharomyces cerevisiae
DjVLGBV Dugesia japonica
CnVAS2 Hydra magnipapillata
CnVAS1 Hydra magnipapillata
PoVAS1 Ephydatia fluviatilis
vasa Gallus gallus
vasa Rana lessonae
vasa Macaca mulatta
vasa Homo sapiens
vasa Bos Taurus
vasa Mus musculus
vasa Rattus norvegicus
vasa Danio rerio
vasa Leucopsarion petersii
vasa Monopterus albus
vasa Ciona savignyi
vasa Ciona intestinalis
vasa Polyandrocarpa misakiensis
vasa Platynereis dumerilii
vasa Botrylloides violaceus
BS-vasa Botryllus schlosseri
vasa Botryllus primigenus
belle Drosophila melanogaster
DjVLGA Dugesia japonica
CnPL10 Hydra magnipapillata
BS-PL10 Botryllus schlosseri
PoPL10 Ephydatia fluviatilis
PL10b Platynereis dumerilii
PL10a Platynereis dumerilii
DDX3 Danio rerio
PL10 Rana lessonae
PL10 Xenopus laevis
DDX3Y Mus musculus
Pongo abelii
DDX3Y Homo sapiens
DDX3Y Pan troglodytes
DDX3 Macaca mulatta
DDX3 Canis familiaris
PL10 D1Pasi Mus musculus
DDX3 Gallus gallus
DDX3 Rattus norvegicus
DDX3 Mus musculus
DDX3 Homo sapiens
0.1

PIWIL2 orthologue, were used at 1:5000 dilutions (unpublished). Our previously developed BS-Pl10 polyclonals (Rosner et al., 2006) were used at 1:4000 dilutions. Anti Oct-4 polyclonals was purchased from Cell Signaling (cat. no. 2750, www.cellsignal.com). Alkaline Phosphatase-conjugated Goat anti-Rabbit IgM or IgG were purchased from SouthernBiotech (cat. no. 4020-04, Birmingham AL, USA) or Jackson ImmunoResearch (cat. no. 111-055-144 West Grove, PA USA), respectively.

A series of experiments are described in the Supplementary section to demonstrate the specificity of the antibodies.

Quantitative PCR

RNA was extracted from tissues by RNAeasy Mini Kit (QIAGEN CA, USA). NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, CA USA) was used to determine RNAs quantities. cDNAs were synthesized with a Verso cDNA kit (cat., AB-1453/B Thermo Fisher Scientific, Epsom, UK). PCR amplifications were performed using Absolute Blue SYBBER Green ROX Mix (cat., AB-4162/B Thermo Fisher Scientific Inc, Epsom, UK) according to manufacturer instructions. We used the 'relative quantification' method developed by Pfaffl (2001), to analyze the results. 18S rRNA (accession no: AB211066) served as the reference gene.

ELISA

Wells of ELISA plates (F96 Maxisorp, immuno plate 442404, Nunc, Denmark) were coated with 50 µl of *B. schlosseri* protein extracts (20 µg/ml diluted in 0.1 M carbonate buffer pH 9.8) and incubated over night at 4 °C. The plates were washed with PBS-T (0.05% Tween 20 in PBS) and post coated with 75 µl of 1% BSA in PBS-T. After an overnight incubation at 4 °C and washing, one of the following 50 µl aliquots was added, rabbit anti-Vasa antibody, rabbit anti Pl10 antibody, or the appropriate pre-immune serums at working dilution, and further incubated for 2 h at 37 °C. After washing, goat anti-rabbit IgG conjugated to alkaline phosphatase (no. 111 055 144 Jackson Research Laboratories, PA, USA; 40 µg/l) diluted in blocking solution was added to each well and incubated for 1 h at 37 °C.

After washing, 50 µl of substrate for alkaline phosphatase, p-nitrophenyl phosphate (pNPP S0942, sigma) was added to each well at a concentration of 1 mg/ml and further incubated for 2 h at 37 °C followed by over night incubation at 4 °C. The OD was determined using ELISA reader (TEcan spectra Image) at 405 nm. Each ELISA result was expressed as a ratio of positive OD (P; the OD obtained by the appropriate specific antibody) and mean absorbance in negative (N; OD obtained by the appropriate pre-immune serum) as previously described (Lapidot et al., 1995).

Western blot analysis

Western blot analysis was performed as described by Rosner et al. (2006).

In situ RNA hybridization

In situ RNA hybridization was carried out with DIG labeled RNA probes. Detection of the BS-Vasa mRNA was done by one of two probes from the 3′ region of the gene, a 230-base probe representing amino acids 593–664 and a 1100-base probe extending on both, a coding region (amino acids 454–664) and the 3′ UTR of the gene. For BS-Pl10 mRNA detection, we used a 576-base probe encoding for 110 aa at the C-terminus gene, and for the 3′ UTR. No significant similarity was assigned to BS-Pl10 and BS-Vasa probes, so each is specific only to the gene from which it derived. Hybridization at 60 °C was performed as previously described (Breitschopf et al., 1992; Rosner et al., 2007).

Hematoxylin–eosin and immunohistochemistry staining

Hematoxylin–eosin and immunohistochemistry staining were performed as described (Rosner et al., 2007) with the following modifications: in some of the experiments, the secondary antibody used to visualized specific interactions was Cy™ 3-conjugated Goat anti-rabbit (Jackson ImmunoResearch laboratories cat. no. 111-165-003) with excitation frequency around 550 nm and emission peak at 570 nm. In these experiments the cover slips were glued by Fluoromount-G™ (SouthernBiotech Birmingham, AL, USA, cat. no. 0100-01).

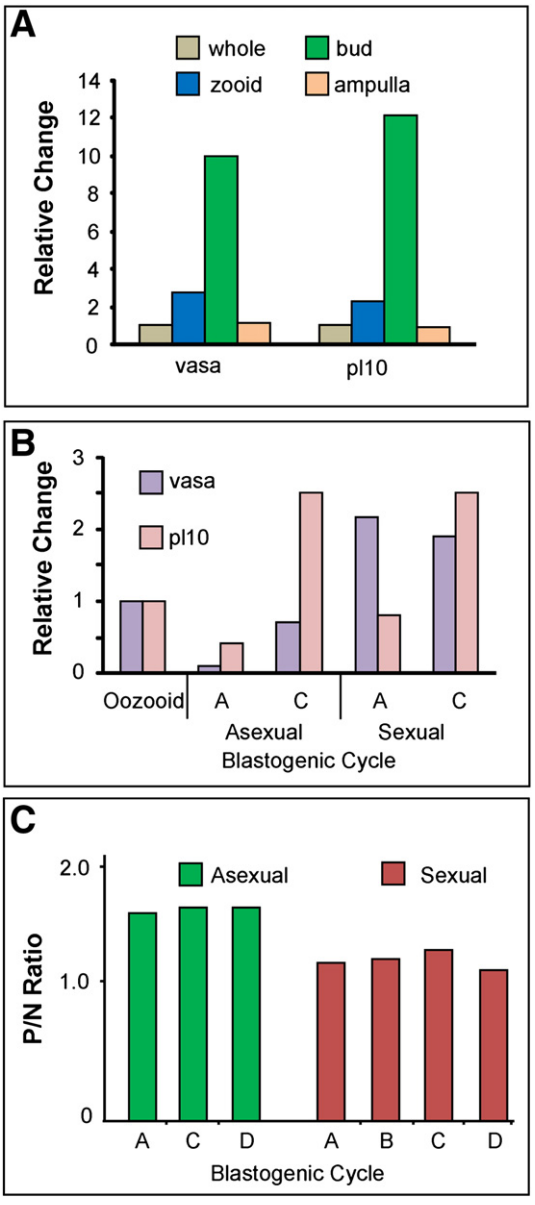


Fig. 2. Quantification of BS-Vasa and BS-Pl10 expressions in *Botryllus schlosseri* various body components, blastogenic stages and sexual maturity. (A) The relative qPCR expression of BS-Vasa and BS-Pl10 mRNAs in various bodily components of *Botrylus schlosseri*. Comparisons were performed on expressions of each gene in zooids, buds, and ampullae, relative to expressions in the whole colony. (B) The relative qPCR expression of BS-Vasa and BS-Pl10 mRNAs in colonies at different blastogenic stages and sexual reproduction status. Values were compared with mRNAs from oozooids of up to 1 week old. (C) Comparison of normal BS-Vasa protein expressions in ramets of different sexual states and blastogenic stages. Asexual colonies do not possess gonads, in contrast to sexual colonies. The protein quantity is expressed as P (positive)/N (negative) ratio.

DAPI staining

DAPI (4′,6-Diamidino-2-phenylindole, Sigma-Aldrich cat. no. D9564) is a highly sensitive fluorescent dye used for detecting DNAs. It has an excitation frequency of 365 nm and emission peak at 450 nm. Staining of nuclei was performed as described by Russell et al. (1975).

Regeneration

Regeneration assays were performed as described by Voskoboynik et al. (2007). Zooids and buds were surgically removed from colonies at blastogenic stages C or D. Formation of new buds from the remaining vasculature was monitored for seven consecutive days following surgery. Samples were fixed and analyzed immunohistochemically.

UVB and UVC irradiation

Colonies were subcloned into ramets 2–3 weeks before irradiation. We used an UVB lamp (VL-6M, 16 W tube, a peak wavelength at 312 nm, power 12 W; Vilber Lourmat, Marne La Vallée, France) that emitted radiation of 16.2 kJm^{-2}/1 h (Rinkevich et al., 2005). Normal sun UVB radiation at midday is 2.9 kJm^{-2} (during 1 h). Ramets were irradiated for 0.5 h, 1 h, 2 h, or 4 h, representing energy of 8.1, 16.2, 32.4, and 64.8 kJm^{-2}, respectively and then harvested. UVC irradiation was performed by Stratagene UV Crosslinker (Model 1800)

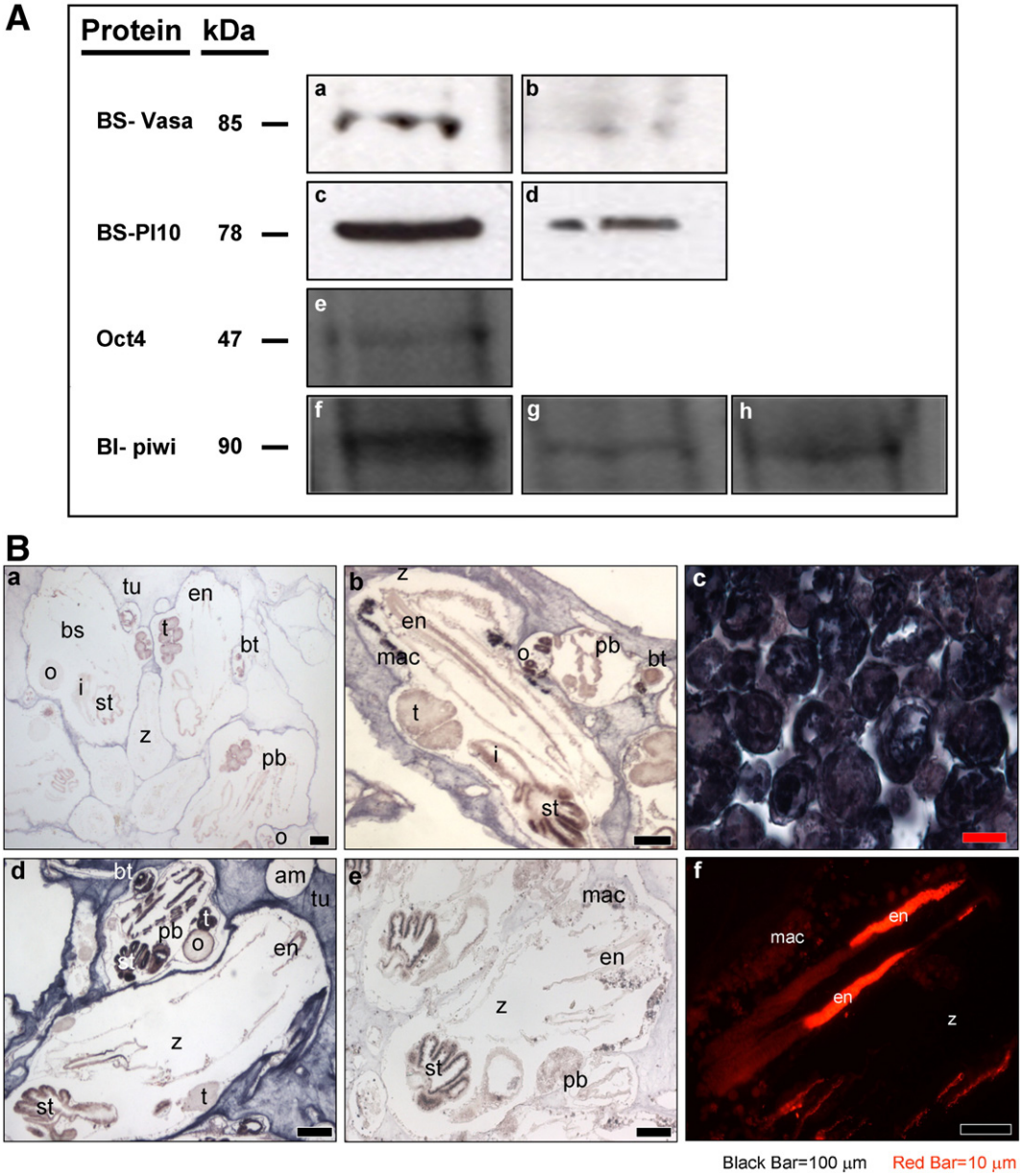


Fig. 3. Expression patterns of selected genes in *Botryllus schlosseri* colonies ongoing sexual reproduction. (A) Western blot analyses performed on *B. schlosseri* buds (a) and whole colony (b) extracts using BS-Vasa antibodies; on *B. schlosseri* buds (c) and zooids (d) extracts using BS-Pl10 antibodies; on *B. schlosseri* whole colony (e) extracts using commercial anti Oct4 antibodies; and on *Botrylloides leachi* (f) and *B. schlosseri* (g–h) whole colony protein extracts using Bl-piwi antibodies. (B) *In situ* expression analyses of mRNA or protein of the selected genes. (a) BS-Vasa mRNA expression in a blastogenic stage D colony. (b) BS-Vasa protein expression in a blastogenic stage B colony. (c) High magnification of the macrophage-like cells near the endostyle stained with BS-Vasa antibodies. (d) BS-Pl10 protein expressions in a blastogenic stage C colony. (e) Bl-piwi protein orthologue expression in a blastogenic stage C colony. (f) Oct4 protein orthologue expression in a blastogenic stage C colony. Abbreviations: am—ampulla; bs—branchial sac; bt—budlet; en—endostyle; i—intestine; mac—macrophage-like cells; o—oocyte; pb—primary bud; st—stomach; t—testis; tu—tunic; z—zooid.

as source. Ramets were irradiated with 40 Jm^{-2} at wavelength of 254 nm during 5 s and then harvested 10 min, 0.5 h, 1 h, 2 h, 4 h, and 24 h after irradiation. Analyses were performed by mRNA relative quantification (qPCR), protein quantification analysis (ELISA), or by immunohistochemistry. Irradiation impacts were evaluated on genet and blastogenic stage levels, so that the controls and the treated ramets in each set of experiment were identical in respect to genet, age, blastogenic stage, and reproductive status.

Results

Isolation and bioinformatic analysis of BS-Vasa

We isolated a 2050-base fragment, a partial fragment from the 3′ part of the gene, whose deduced protein encompasses 547 aa (out of 687 aa in *Botryllus primigenus*) of the BS-Vasa gene. This BS-Vasa sequence (accession no: FJ455513) contained the DEADc motif typical of DEAD-box RNA helicases, the HELICc motif associated with DEXDc-, DEAD- and DEAH-box proteins and a Zinc knuckle motif (NCBI nomenclature of conserved domains), known to be involved in eukaryotic gene regulation. The presence a Zinc knuckle motif in the BS-Vasa is typical of the invertebrate orthologues, not found in any vertebrate.

NCBI blast analysis of deduced BS-Vasa protein sequence demonstrated its highest homology (*E* value 0.0, 63–78% identity) to other Vasa orthologues isolated from tunicates like *B. primigenus*, *Polyandrocarpa misakiensis*, *Botrylloides violaceus*, *Ciona savignyi* and *Ciona intestinalis*. Comparison of BS-Vasa mRNA with the sequence of BS-Pl10 mRNA revealed low similarity (86% identity over a 44-base fragment, while the entire fragment is of is of 2050 bp). However, protein sequence comparisons of these genes revealed 50% identity over a fragment of 481 aa, whereas most prominent divergence occurred at the 5′ and 3′ ends, as expected.

Phylogenetic relations were established between BS-Vasa, BS-Pl10 proteins and some of their nearest orthologues, to confirm their classification as Vasa or Pl10 orthologues, respectively (Fig. 1B). The analysis compared between 380 aa fragments of these proteins, which contained conserved motifs of the DEAD-box family. The phylogenetic tree was constructed via a two-step procedure. The first step used PROTTEST (Abascal et al., 2005) to decide upon the best model for our sequences. This analysis suggested JTT+I+G as the model of choice. Then, we used these data to reconstitute a phylogenetic tree with the web based package "Tree-Puzzle" (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=puzzle), which performs maximum likelihood analysis. The analysis demonstrated that BS-Vasa and BS-Pl10 are clustered, each with its respective orthologues (BS-Vasa with other members of the Vasa subfamily and BS-Pl10 with members of the DDX3 subfamily), hence BS-Vasa and BS-Pl10 are paralogues to each other. Another interesting outcome from this analysis was the clustering of the planarian *Dugesia japonica* gene, DjVLGA, with the Pl10 subfamily and not with Vasa as had been previously thought. Following this new classification, the expression of DjVLGA in somatic cells (Shibata et al., 1999) is in agreement with Pl10 distributions and functions.

Quantification of BS-Vasa and BS-Pl10 gene products

Because of the sequence similarity of the Vasa- and Pl10-related proteins and the partial overlapping of their expression patterns reported in the literature (Shibata et al., 1999), we chose to compare their exact mRNA and protein expressions in *B. schlosseri*'s bodily compartments. We quantified either BS-Vasa or BS-Pl10 mRNAs by using the relative qPCR quantification technique, with Pfaffl (2001) equations analyses. Additionally, all expressions were compared on the genet level to reveal colony specific variations. mRNA expressions in buds, zooids and ampullae were compared for each specific gene expression (BS-Vasa, BS-Pl10) to corresponding whole colony expressions. The values obtained for 'whole colonies' were considered as 'control' in Pfaffl's equations. Three genotypes were analyzed and the results for one genet are depicted in Fig. 2A. Both, BS-Vasa and BS-Pl10 mRNAs' levels were significantly elevated in buds relative to whole animals: 12–108 fold in BS-Pl10 mRNA (the genotype specific elevation of 12 fold is depicted in Fig. 2A) and 10–14 fold for BS-Vasa mRNA (the genotype specific elevation of 10 fold is depicted in Fig. 2A). In both genes, the expressions in zooids increased by two fold while the expressions in ampullae were similar to entire animal's level (Fig. 2A).

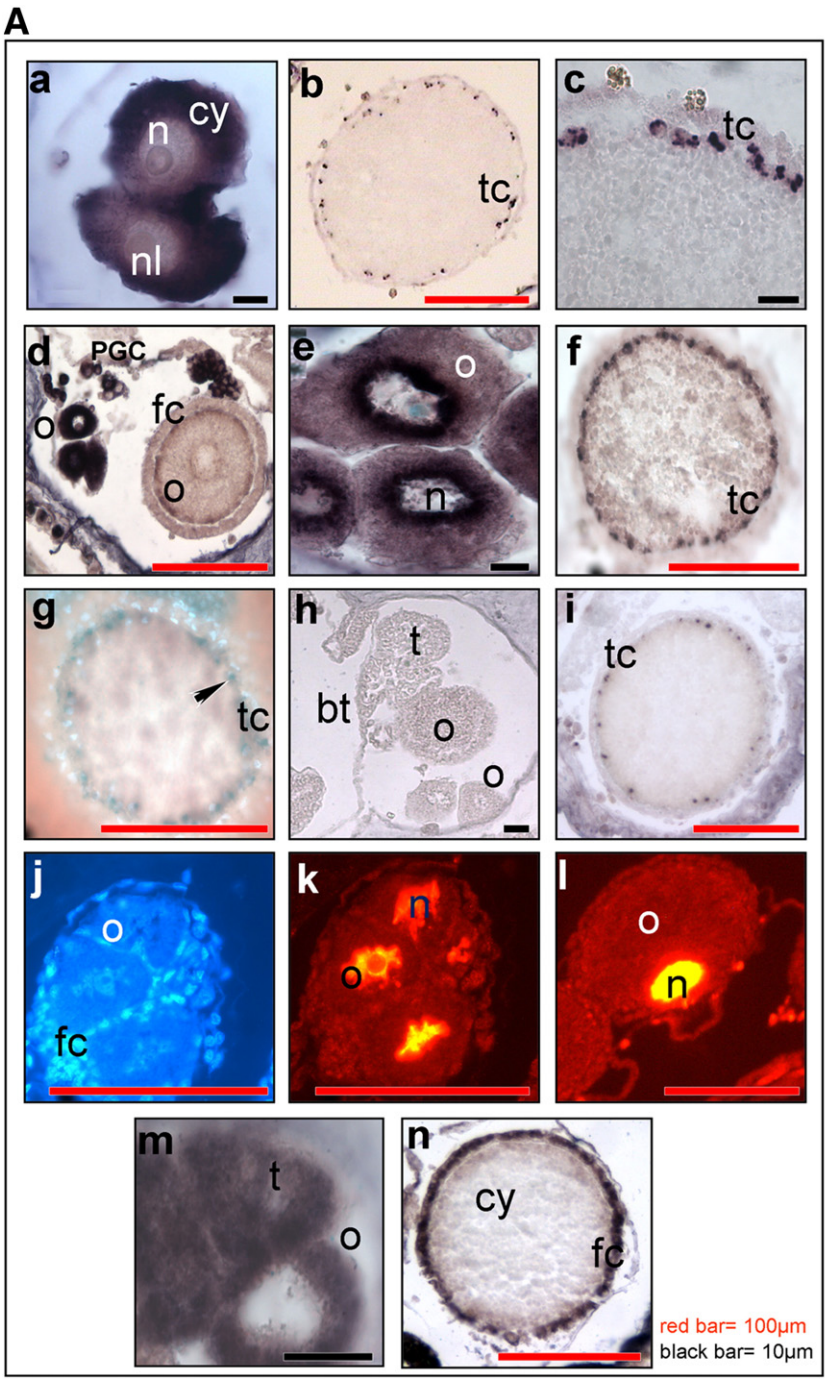
Next, we compared entire animal expressions of BS-Vasa and BS-Pl10 mRNAs at different reproductive states and two representative blastogenic stages ('A' and 'C'; sensu Mukai and Watanabe, 1976a). For this analysis, mRNA extracted from a pool of oozooids served as 'control'. The levels of BS-Vasa mRNA were higher in oozooids compared to asexual animals at both blastogenic stages and were almost doubled in sexually reproducing animals. BS-Pl10 mRNA levels, however, were elevated in blastogenic stage C colonies, either sexually matured or asexual colonies (Fig. 2B).

BS-Vasa protein quantification was performed by ELISA, comparing the expressions in asexual and sexual animals at different blastogenic stages (Fig. 2C). Low P/N ratios, close to one, indicate low quantities of the BS-Vasa protein. Changes even as small as a few tenths of a fold, represent a true difference, as demonstrated in other systems (Lapidot et al., 1995). Whereas no correlation was recorded with any blastogenic stage, it is interesting to note the higher levels of BS-Vasa protein in young asexual colonies relatively to sexually matured colonies. Since sexual and asexual colonies are demarcated by the presence of gonads, the higher quantities of BS-Vasa in asexual colonies may point to either increased number of germ cells dispersed within the colony soma and/or to the expression of BS-Vasa in tissues other than germ cells. The differences between the BS-Vasa mRNA and protein expression patterns may indicate multi-level expression regulation.

Identification of BS-Vasa and BS-Pl10 proteins and of Bl-piwi and Oct4 orthologues in B. schlosseri

BS-Vasa antibodies identify a 85 kDa protein which is enriched in buds isolated from sexual colonies (Fig. 3Aa) relative to the whole colony (Fig. 3Ab). BS-Pl10 antibodies, as previously described (Rosner et al., 2006), recognize a band of 78 kDa enriched in buds (Fig. 3Ac), relative to corresponding functional zooids (Fig. 3Ad). Anti Oct 4

Fig. 4. (A) BS-Vasa, BS-Pl10, Bl-piwi and Oct4 expressions during germ cell differentiation—oogenesis. (a) Young and (b) mature oocytes labeled with BS-Vasa mRNA probe. (c) High magnification of a mature oocyte labeled with BS-Vasa mRNA probe. Test cells show positive response. (d–f) Oocytes at various stages of development stained with BS-Vasa antibodies. (g) A mature oocyte double stained with DAPI and BS-Vasa antibodies. (h–i) Oocytes stained with Bl-piwi antibodies. A section showing several oocytes double stained with (j) DAPI and (k) Oct4 antibodies. (l) Mature oocytes stained with anti-Oct4 antibodies. (m) Young and (n) mature oocytes stained with BS-Pl10 antibodies. The follicular layers in the mature oocytes are heavily stained. (B). BS-Vasa, BS-Pl10, Bl-piwi and Oct4 expressions during germ cells differentiation—spermatogenesis. Testes located in primary buds contain early staged male cells; testes in zooids contain mature male cells. (a) Young or mature (b) testes stained with BS-Vasa mRNA probe. (c) Mature and (c–d) young testes reacted with BS-Vasa antibodies. (e) High magnification of a young testis showing BS-Vasa protein expression within the cytoplasm. (f) A young testis stained with BS-Pl10 antibodies and (g) high magnification of same section demonstrating that the expressions are within the cytoplasm. (h) A young testis stained with anti-Oct4 antibodies. The visualization of primary antibody binding was performed with either anti-rabbit-alkaline phosphatase or with anti-rabbit cy3 coupled secondary antibody. Abbreviations: bt—budlet; cy—cytoplasm; e—embryo; fc—follicular cells; n—nucleus; nl—nucleolus; o—oocyte; PCG—premordial germ cells; t—testis; tc—test cells; z—zooid.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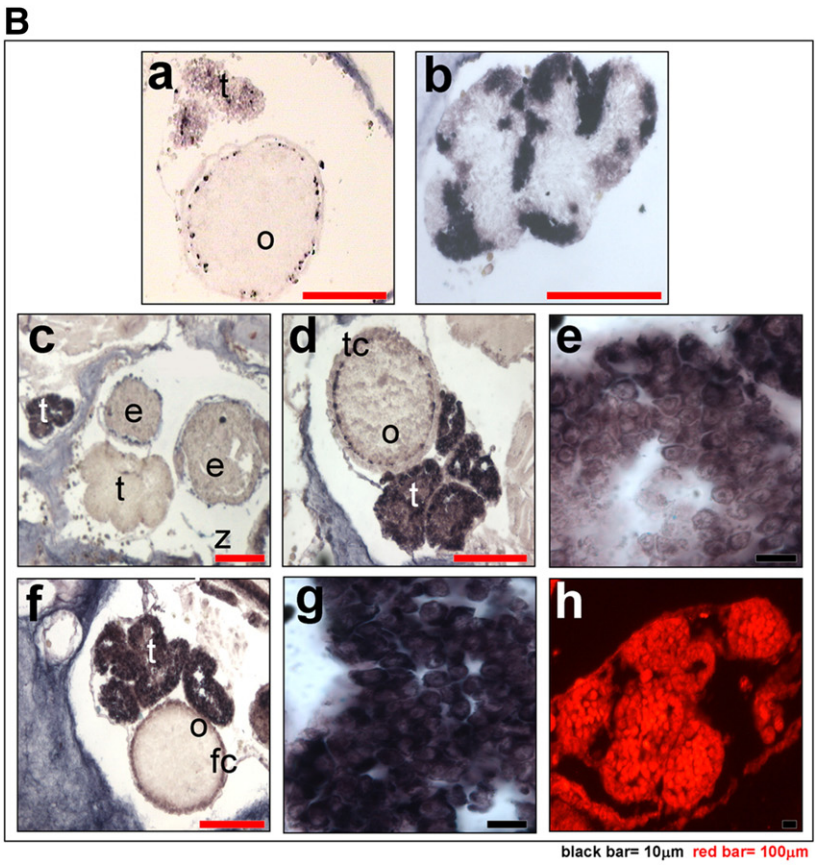
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antibodies recognize a band of 47 kDa (Fig. 3Ae), which reflects the size range specified by the commercial supplier. Finally, Bl-piwi antibodies recognize a 90 kDa protein in *B. leachi* (Fig. 3Af) as well as in two protein extracts from *B. schlosseri* colonies (Fig. 3Ag and h). This protein size fits the size range of piwi members as commercial anti-piwi antibodies visualize them (for example, Abcam's piwi antibody cat. #ab5207).

In situ mRNA and protein expressions of BS-Vasa and BS-Pl10, and of Bl-piwi and Oct4 orthologues

BS-Vasa mRNA was markedly expressed in germ cells (except of mature oocytes), in embryos, test cells, in subsets of blood cells and less distinctively expressed in bud soma tissue and the zooid's stomach (Fig. 3Ba). BS-Vasa protein was detected in young germ cells within primary buds (Fig. 3Bb), in some blood cells, test cells, macrophage-like cells around the endostyle (Fig. 3Bc) and in zooidal stomach (Fig. 3Bb). In other tissues expressing high BS-Vasa mRNA (bud's soma, the zooidal testes and embryos), BS-Vasa protein levels were low (Fig. 3Bb). Results of BS-Pl10 protein expression patterns (Fig. 3Bd) and mRNA (data not shown) confirmed earlier results (Rosner et al., 2006, 2007) for overlapping mRNA and protein expressions. High BS-Pl10 expressions were detected in buds' tissues, in young germ cells (both oocytes and spermatogonia) and in several embryonic tissues. Similar to BS-Vasa, BS-Pl10 protein was found elevated in the macrophage-like cell population around the endostyle (Fig. 3Bd).

Bl-piwi antibodies were developed against epitopes of the botryllid ascidian congener species *B. leachi* (Rinkevich Y., unpublished). Immunohistochemical analysis (Fig. 3Be) revealed intense staining of the macrophage-like cells in the endostyle area, test cells, tunic cells and cells of the digestive system, mainly in the stomach. In contrast to the expression pattern in other metazoans (Tan et al., 2002), Bl-piwi orthologue expression in gonads was faint (Fig. 3Be). Oct-4 (Fig. 3Bf) heavily stained areas of the zooidal endostyle supporting the suggestion of the endostyle being a stem cell niche (Voskoboynik et al., 2008). Additionally, Oct-4 stained oocytes, embryos and some of the cells of the branchial sac, but did not stain the macrophage-like cells around the endostyle.

BS-Vasa, BS-Pl10, Bl-piwi and Oct4 expressions during oogenesis

Detailed analyses of *in situ* mRNA and protein expressions for above marker genes in germ cells were performed during gametogenesis (Manni et al., 1994). BS-Vasa mRNA (Fig. 4Aa–c) and protein (Fig. 4Ad–g) showed similar expression patterns in young and mature oocytes. Both methods stained 6–12 μm granulated Vasa positive cells in the gonad sac (Fig. 4Ad). In more developed oocytes (40 μM) with large, prominent nuclei, high levels of BS-Vasa mRNA and protein were evenly distributed in the cytoplasm (Fig. 4Aa and d, respectively). At late oogenesis, BS-Vasa protein accumulated at the perinuclear cytoplasm (Fig. 4Ae) as reported in other animals (Rebscher et al., 2007), but disappeared towards the end of oogenesis and ovulation (Fig. 4Ab and f, respectively). At this stage, a monolayer of cells (6–7 μm; Fig. 4Ac) situated at the oocytes' periphery, just beneath the follicular layer (Manni et al., 1993), expressed high levels of both BS-Vasa mRNA and protein (Fig. 4Ab–c, f–g). Double staining with DAPI and anti-Vasa antibody, documented the cellular identity of the BS-Vasa expressing monolayer (Fig. 4Ag, arrow). Based on their location between the oocyte and the follicular layers, the BS-Vasa positive cells were identified as 'test cell' (Zaniolo et al., 1989).

Oocytes at different differentiation stages did not stain with anti Bl-piwi antibodies, indicating that oocytes do not express Bl-piwi orthologue (Fig. 4Ah, i). Yet, the test cells around mature oocytes expressed Bl-piwi (Fig. 4Ai). Commercially available anti Oct4 polyclonals stained oocytes' nuclei of all development stages, including mature oocytes (Fig. 4Ak, l). Double stained tissue sections with DAPI (Fig. 4Aj) and fluorescent anti Oct4 antibody (Fig. 4Ak) demonstrated that Oct4 is specifically expressed in the nucleus of oocytes and not in the follicular cell layers or in the test cell layer.

Young oocytes specifically expressed high levels of BS-Pl10, which decreased as they matured (Fig. 4Am–n; Rosner et al., 2006). BS-Pl10 expression in young oocytes, as BS-Vasa protein expression, was concentrated in the cytoplasm. Following oocytes maturation, the concentration of the protein decreased, evenly distributed in the cytoplasm. In contrast to BS-Vasa, BS-Pl10 was highly expressed in the follicular cells, wrapping the mature oocyte (Fig. 4An), but not in test cells.

BS-Vasa, BS-Pl10, Bl-piwi and Oct4 expressions during spermatogenesis

Moderate expressions of BS-Vasa mRNA were recorded in young testes situated in the primary buds (Fig. 4Ba); they were significantly lower than expressions detected in the more developed male cells in the periphery of matured testes within the zooids (Fig. 4Bb). In mature sperm cells, at the centers of the testes, BS-Vasa mRNA levels were the lowest. BS-Vasa protein showed the opposite pattern, high expression in young testes within buds (Fig. 4Bc–d) and low, evenly distributed expression, in the zooids' mature testes (Fig. 4Bc). BS-Vasa protein was located in the cytoplasm of the highly expressing male cells (Fig. 4Be). BS-Pl10 mRNA and protein expressions showed similar patterns, resembling the BS-Vasa protein expression (Fig. 4Bf–g), i.e. high expression in the cytoplasm of young male cells and low expression in mature sperm cells. Oct4 protein levels that were moderately expressed in young spermatic cells dropped in mature sperm (Fig. 4Bh). Bl-piwi protein was not expressed in any sperm precursors or in mature sperm (Fig. 4Ah).

Expressions in embryos

B. schlosseri embryos (Fig. 5A) possessed a high number of evenly spread Oct4 positive cells in body trunk and tail (Fig. 5B). Staining of embryos (early tail-bud stage) with BS-Vasa mRNA probes revealed similar distribution of many positively stained cells mainly in the body trunk (Fig. 5C). However, most of these cells did not express BS-Vasa protein (Figs. 5D, F). Staining of embryos at the gastrula stage with anti BS-Vasa antibodies (Fig. 5D) revealed weak, even staining throughout the embryo (Fig. 5D) and strong expression in a population of 9–12 μm test cells situated between the embryo soma and the inner follicular layer (Fig. 5D, black arrows; Fig. 5E). These test cells continued to express high levels of BS-Vasa in subsequent stages of embryonal development as they migrated into the tunic around the embryo (Fig. 5F). In parallel, we identified a small population (3–5 cells per embryo) of 6–7 μm BS-Vasa protein positive cells in wrapped-tail stage embryos, situated in the center of the body trunk (Fig. 5F, red arrows; Fig. 5G). This pattern of BS-Vasa mRNA and protein expressions are different from those observed in the solitary ascidian as described first by Fujimura and Takamura (2000), Takamura et al. (2002) and further elaborated by Shirae-Kurabayashi et al. (2006). There, a preformistic sequestering has been described, showing CiVH (the Vasa orthologue) mRNA accumulation in a specific region of the fertilized one cell stage embryo. In 64-cell stage embryo, CiVH protein was concentrated in B7.6 cells, and redistributed in the subsequent development to two discrete cell masses of the tail. Following hatching, these CiVH positive cells migrated back to the trunk, along a specific tissue, to create the primitive gonad. Here we demonstrate that BS-Vasa mRNA is evenly distributed in many cells, most of which are located the body trunk till early tail-bud stage, and that BS-Vasa protein is still evenly distributed at gastrula stage and thus may represent epigenetic mode of differentiation. These two different modes of differentiation culminate in formation of gonads at different stages of development.

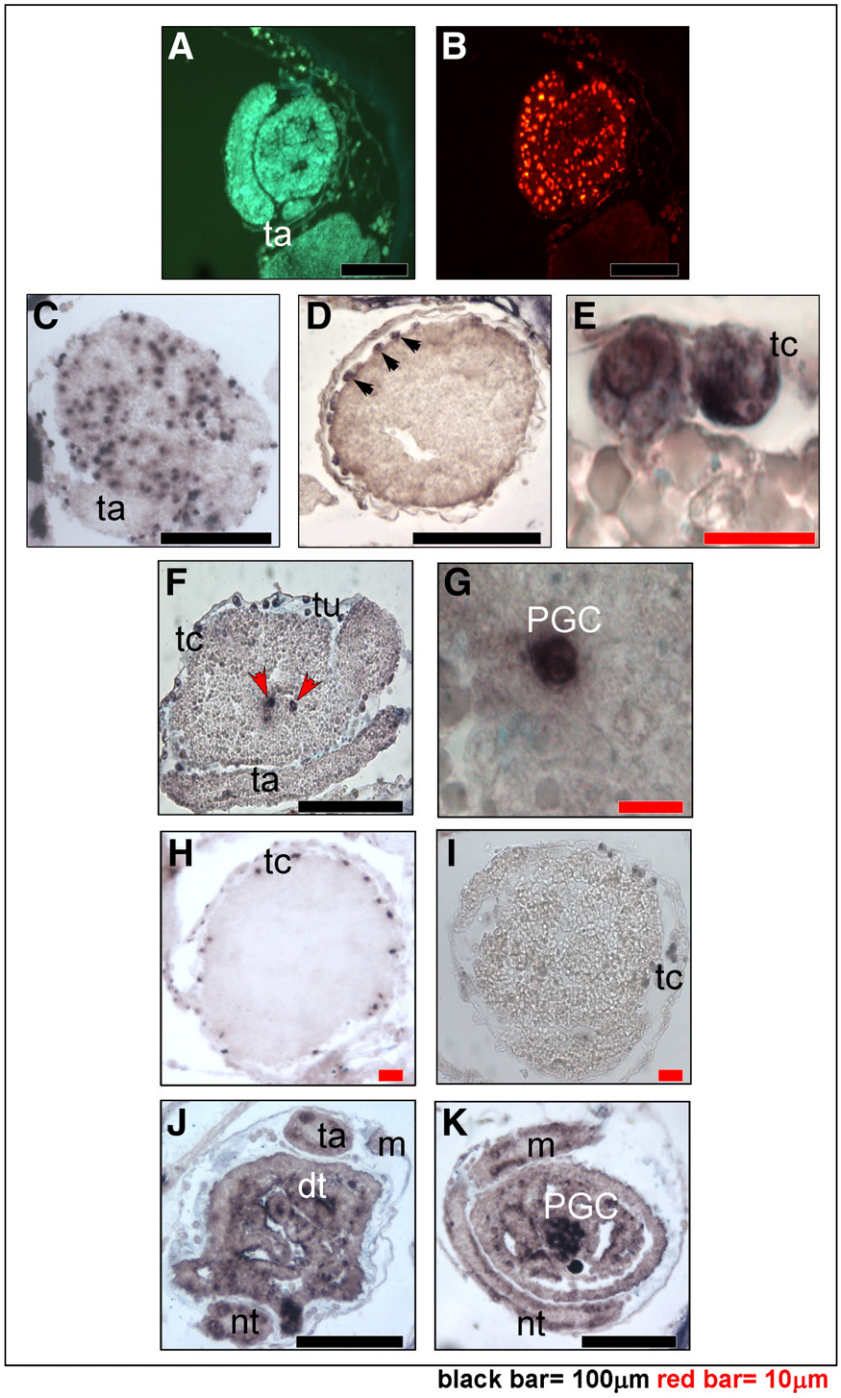

Fig. 5. *Botryllus schlosseri* histological sections of gastrula or wrapped-tail stage embryos stained with BS-Vasa, BS-Pl10, Bl-piwi and Oct4 antibodies. (A) Auto-fluorescence emission (360–370 nm) or (B) immunohistochemical staining with Oct4 antibodies of the same section from a wrapped-stage embryo. (C) *In situ* mRNA hybridization of a tail-bud staged embryo stained with BS-Vasa mRNA probe; (D) immunohistochemical analysis with BS-Vasa antibodies of an embryo at gastrula stage with, (E) emphasis on the test cell region. BS-Vasa protein staining of (F) wrapped-staged embryo in which test cells are stained within the embryonic tunic. Black arrows point towards the test cells and red arrows point towards the PGC-like cells. (G) High magnification of BS-Vasa positive PGC-like cells in a wrapped-tail embryo; Immunohistochemical analysis with Bl-piwi antibodies of (H) gastrula, or (I) wrapped-tail stage embryos; (J–K) immunohistochemical analysis of an embryo stained with BS-Pl10 antibodies. Abbreviations: dt—digestive tract; m—muscle; nt—notochord; PGC—PGC-like cells; ta—tail; tc—test cells; tu—tunic.

Staining of embryos on the gastrula (Fig. 5H) or wrapped-tail stages (Fig. 5I) with Bl-piwi antibodies revealed staining of the test cells in both stages, very similar to BS-Vasa expressions. Unlike the BS-Vasa staining pattern, no other cell types were Bl-piwi positive. Staining of embryos with anti BS-Pl10 antibodies (Fig. 5J) confirmed previously documented staining of the muscles around the notochord and digestive system (Rosner et al., 2006). Additionally, few cells of 6–7 μm were stained in the middle of the body trunk (Fig. 5K).

Expressions in buds and budlets

Budlets (secondary buds) at blastogenic stage B were visualized in histological sections by two methods: (1) auto-fluorescence emission through illumination with UV (Fig. 6A) and (2) staining with BS-Vasa antibodies (Fig. 6B). Two distinctive BS-Vasa positive microenvironments were revealed. The first was the gonad rudiment (Fig. 6A), containing dozens of BS-Vasa^{+} cells, including small 6–12 μm cells

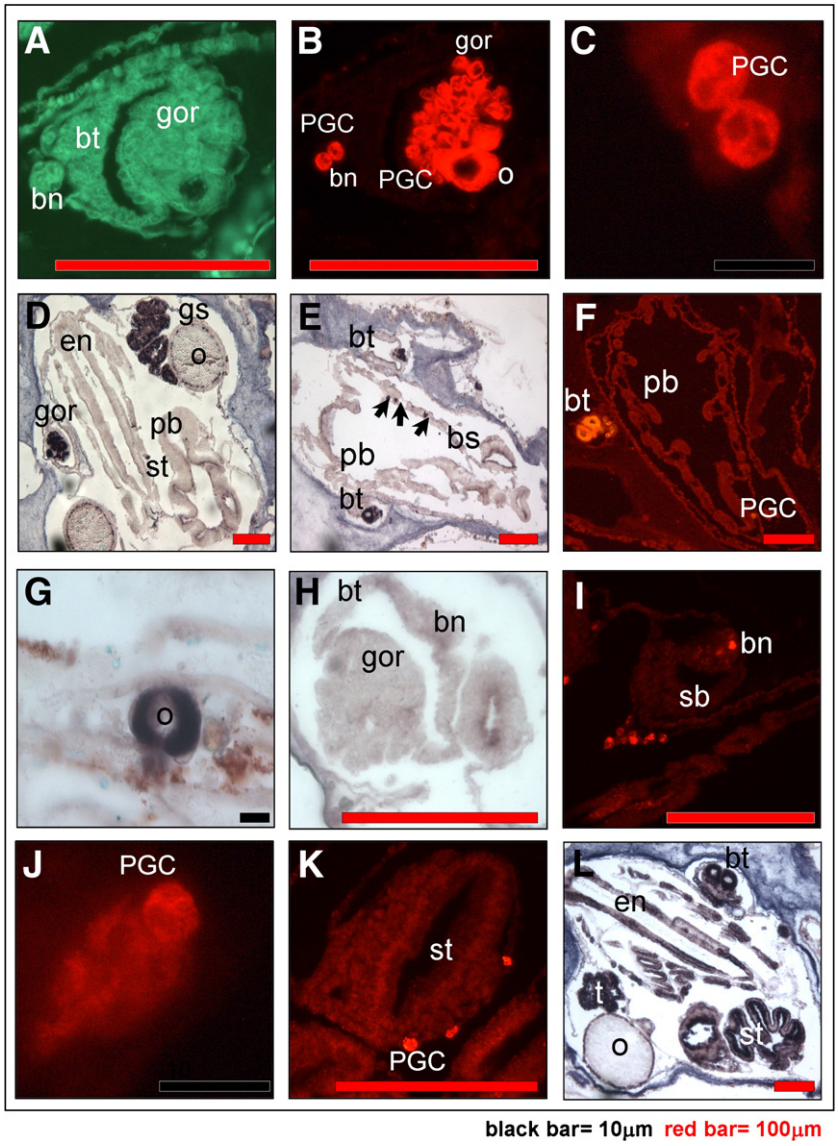

Fig. 6. Primary buds and budlets as important germ cell hubs. (A) an auto-fluorescent planar view (360–370 nm) of histological section showing a whole budlet; (B) the same section stained immunohistochemically with BS-Vasa antibodies, demarcating the budlet niche and gonad rudiment. (C) High magnification of PGC-like cells stained with BS-Vasa antibodies at the budlet niche; primary buds at blastogenic stage C stained with BS-Vasa antibodies revealing the (D) gonad rudiment, the gonad sac and (E–F) PGC-like cells intercalated in bud's tissue; (G) an oocyte in the blood vessel stained with BS-Vasa; (H) budlet stained with Bl-piwi antibodies revealing negative responses at the budlet niche and gonad rudiment; Staining with Oct4 antibodies reveal positive responses at (I) budlet niche, within (J) PGC-like cells and (K) free PGC-like cells within primary buds; (L) Positive responses are detected in the gonad rudiment, gonad sac and gonad niche after staining with BS-Pl10 antibodies, as well as in soma cells. Abbreviations: bn—budlet niche; bs—branchial sac; bt—budlet; en—endostyle; gs—gonadal sac; gor—gonad rudiment; o—oocyte; pb—primary bud; PGC—PGC-like cells; sb—secondary bud (budlet); st—stomach; t—testis.

and early staged oocytes of different sizes. Gonad rudiments were formed only in budlets of sexual colonies, developing to gonads (Fig. 6D) at blastogenic stage C. The second microenvironment (Figs. 6A, B) situated distal to the gonad rudiment, is described here for the first time and termed as the 'budlet niche'. The 'budlet niche' found in asexual and sexual colonies, housed 2–5 small 6–7 μm granulated (Fig. 6C) and BS-Vasa positive cells. In addition, BS-Vasa^{+} cells (6–12 μm) were intercalated with the branchial sac tissues (Fig. 6E, arrows) in the stomach (Fig. 6F,) and in the common blood vessels. BS-Vasa^{+} germ cells also circulated freely in the animal vasculature (an example of a circulating 25 μm oocyte; Fig. 6G). While Bl-piwi antibodies stained neither the gonad rudiment nor the budlet niche (Fig. 6H), Oct4 staining showed cells of about 6–12 μm similar to the BS-Vasa positive cells at the 'budlet niche' (Figs. 6I, J). Additionally, Oct4 stained 6–12 μm cells sporadically located within the primary buds (Fig. 6K).

We propose that these BS-Vasa granulated (Fig. 6C) and Oct4 (Fig. 6I) positive cells, detected in the budlet niche and blood vessels in sexual and asexual colonies alike, are primordial germ cells (PGC). These PGC-like cells are found also in the gonad rudiment. BS-Pl10 protein staining revealed the PGC-like cells at the budlet niche (Fig. 6L), oocytes differentiating within the gonad rudiment (Fig. 6L) and differentiating sperm and oocyte precursors within the gonadal space (Fig. 6L). However, since BS-Pl10 stained heavily the somatic tissues of the buds and budlets as well, it is ineffective in differentiating between germ and soma cells.

BS-Vasa expressions in oozooids and asexual young colonies

We analyzed BS-Vasa protein expressions in oozooids (Fig. 7A, arrow) and blastozooids of young, asexual individuals (Fig. 7B, arrow), developed from oozooids. In these preparative, 3–5 PGC-like BS-Vasa^{+}

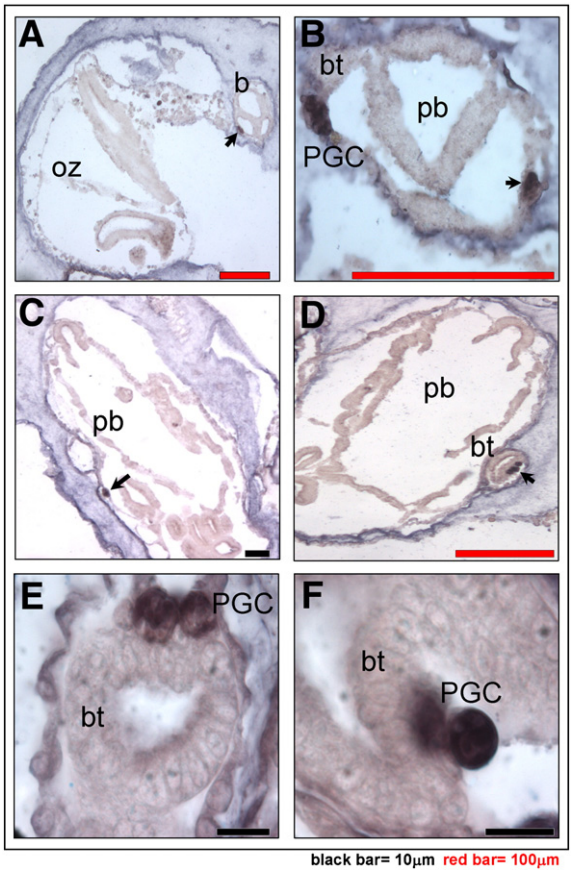

Fig. 7. PGC (primordial germ cell) niches in oozooids and asexual colonies. Immunohistochemical analysis of (A) oozooid (B) blastozooids and (C–F) buds from young asexual colonies of *B. schlosseri* (C–F) stained with BS-Vasa polyclonals. Arrows point to PGC-like cells. Abbreviations: b—bud; pb—primary bud; bt—budlet; oz—oozooid; PGC—PGC-like cells.

cells were found attached to the bud's atrial epithelium, adjacent to a disc which would develop eventually into a budlet. No other positively stained cells were found in zooidal tissue of young individuals (Fig. 7A). Following several blastogenic cycles, we continuously documented PGC-like cells at similar position proximal to the early staged budlets, at the stage of a thickened disc (Fig. 7B). As budlets developed into the 'sphere' stage, PGC-like cells were found within the budlet niche (Figs. 7D–F). In the primary bud, PGC-like cells were located on atrial epithelium proximal to the region where testes would be in sexually matured animals (Fig. 7C).

Regeneration

Voskoboynik et al. (2007) have recently described whole body regeneration in *B. schlosseri*, initiated from ampullae and peripheral blood vessels fragments isolated at blastogenic stage 'D'. Since BS-Vasa$^+$ cells were recorded circulating in colonial vasculature (Fig. 8A, arrows), it is of special interest to document the existence of BS-Vasa$^+$ cells in regenerative fragments, a phenomenon that may also indicate germ cells neogenesis. For that purpose, we removed zooids, buds, and budlets from 10 sexually mature colonies at blastogenic stages 'C' or 'D' and observed regeneration under a microscope every day for a week. In six preparative, the newly formed buds were fixed and immunohistochemically analyzed with BS-Vasa polyclonals. In four of the six preparative, BS-Vasa$^+$ staining was found in the regenerative buds, restricted to a few 7–8 μm granular Vasa positive cells (Fig. 8B). In three preparative, the BS-Vasa$^+$ cells were intimately contacted with the bud soma (Fig. 8B), whereas in the last case, there was a distance of several microns between the tissue and the BS-Vasa$^+$ cells (Fig. 8C).

Impacts of UV irradiation

To observe the impacts of DNA breakage on Vasa (may result in post-translational modification; Ghabrial and Schüpbach, 1999) and PL10 gene products, we subjected 52 *B. schlosseri* ramets from nine genets to either, brief (5 s) UVC irradiation (Fig. 9Aa), or to longer periods (0.5–4 h) of UVB irradiation (Fig. 9Ab). UVC irradiated colonies were returned to our farming-facility immediately upon irradiation and sampled at fixed intervals. UVB irradiations were performed in duplicates. One duplicate was fixed immediately after irradiation, while the other was returned to the farming-facility for 24 h, before being fixed and analyzed.

Shortly after UVC irradiation, colonies exhibited dilation of blood vessels and accumulation of pigment and other blood cells in ampullae (Fig. 9Aa). Gradually, the irradiated colonies returned to their normal phenotypes, 4–24 h after irradiation. In UVB irradiated ramets, the animals responded during irradiation by opening wide the exhalant siphons and by attenuated blood flow. Ramets exposed to UVB for 2–4 h deteriorated, as already seen 24 h after irradiation (Fig. 9Ab), by enlargement of blood vessels and disruption of ampullae structure (typical to stress conditions; Rinkevich and Weissman, 1990; Rosner et al., 2007). In time, disruption of the normal course of blastogenesis was recorded (Fig. 9Ab), as previously described (Rinkevich and Weissman, 1990; Laird and Weissman, 2004). This disruption included premature apoptosis, unsynchronized blastogenic stages D/A switch, and finally, attenuated absorption of the old generation of zooids (Fig. 9Ab). Hematoxylin–eosin staining revealed moderate changes on the cellular level after short irradiations and major changes in the somatic tissues subjected to 2–4 h UVB irradiation (Fig. 9Ac–d). The grave changes included blood cell

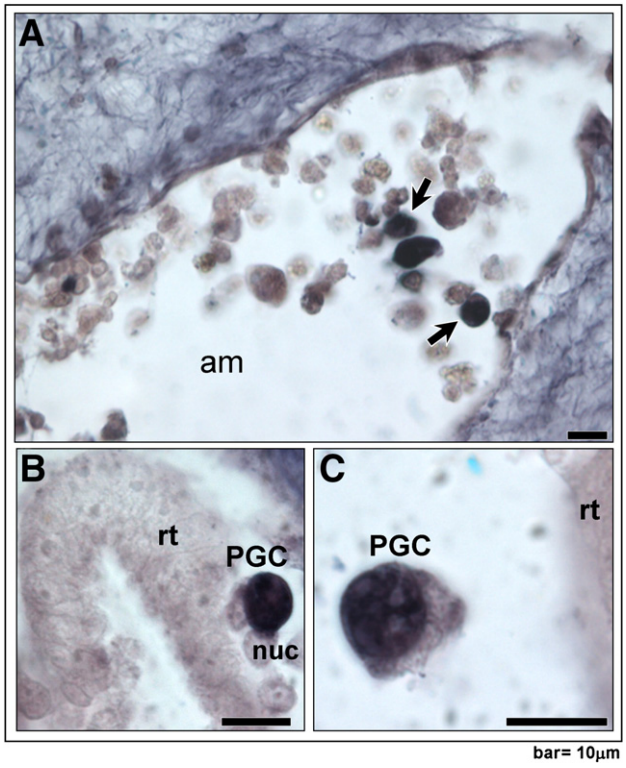

Fig. 8. Regeneration of new zooids from isolated peripheral vasculature of *B. schlosseri*. (A) Immunohistochemical analysis with BS-Vasa antibodies of an ampulla from naive colony; (B and C) similar analysis of regenerating tissues within ampullae following zooids and buds excision. The regenerating tissues do not express BS-Vasa protein, however isolated BS-Vasa positive cells are recruited from vasculature and are detected in (B) contact or (C) close proximity to the regenerating tissue. Abbreviations: am—ampulla; nuc—nursing cells; PGC—PGC-like cells; rt—regenerating tissue. Arrow points towards BS-Vasa positive cells.

hemolysis, disruption of tissue structure and emergence of new two cellular constructions, a basophilic mass of cells in buds (Fig. 9Ac), and some eosinophilic cells around the zooidal endostyle (Fig. 9Ad). Both cell populations appeared after 2 h of irradiation and did not recede after a 24 h of growth in normal conditions. Oocytes (20–30 μm) were observed in all analyzed samples. Testes disappeared after irradiation doses of 1 h or more.

BS-Vasa protein staining was detected in 20–30 μm oocytes (Fig. 9Ae), in PGC-like cells and germ cell precursors in the gonad rudiment and the budlet niche (Fig. 9Af), regardless of the UVB dosage. The basophilic cell populations in buds revealed low BS-Pl10 protein expression and were negative to BS-Vasa protein (not shown). The soma, especially the buds and budlets, appeared as puffy and abnormal (Fig. 9Af and h). Distribution of BS-Pl10 protein staining (Fig. 9Ag–i) was not affected by UVB irradiation, it remained high in buds and gonads and low in zooids (Fig. 9Ag). Like BS-Vasa, BS-Pl10 was also highly expressed in young oocytes (Fig. 9Ag–h) even 24 h after UVB irradiation. Elevated expressions of BS-Pl10 protein were detected in the small cells along zooidal endostyle (Fig. 9Ai), probably the previously described eosinophilic cells.

We further quantified BS-Vasa (Fig. 9Ba) and BS-Pl10 (Fig. 9Bb) protein levels by ELISA on three UVC irradiated genets. All genes showed an immediate short increase (peaks at 10 min for BS-Vasa and 30 min for BS-Pl10) followed by a swift decrease (within 0.5 h after the peak for BS-Vasa and 1–1.5 h after peak for BS-Pl10). BS-Vasa mRNA (Fig. 9Bc) showed similar responses to UVB. It peaked within 0.5 h of exposure and although colonies were still under irradiation, mRNA decreased within the following 0.5 h and reached normal levels during the rest of the irradiation period (4 h). BS-Pl10, mRNA levels increased 0.5–1 h at onset of UVB irradiation (Fig. 9Bd) and decreased within 0.5–1 h following the peak (Fig. 9Bd). Parallel protein expression analyses for BS-Vasa protein expressions demonstrated the same tend. After 24 h of normal conditions, BS-Vasa protein (Fig. 9Be) were elevated in colonies irradiated for 1 h or for longer periods while BS-Pl10 protein levels were inversely correlated with dosage of radiation (Fig. 9Bf), highest in lower UVB treated ramets.

Discussion

Vasa gene products, which have been detected in all studied metazoans (Raz, 2000; Mochizuki et al., 2001) are commonly used as universal germ cell markers in Animalia. Vasa protein is involved in establishing functional germ line and in specification of germ cells (Johnstone and Lasko, 2004), roles that had been suggested even for organisms whose nature, origin, and development of the germ line are still unknown (Raz, 2000; Mochizuki et al., 2001; Brown and Swalla, 2007). As the germ line of *B. schlosseri* has not been identified yet morphologically or molecularly and its homing locations are still elusive (Akhmadieva et al., 2007), we isolated and sequenced BS-Vasa, the *B. schlosseri* orthologue of Vasa, as molecular marker for primordial germ cell (PGC) fate. To distinguish between Vasa and the closely related Pl10 protein subfamily already studied in this

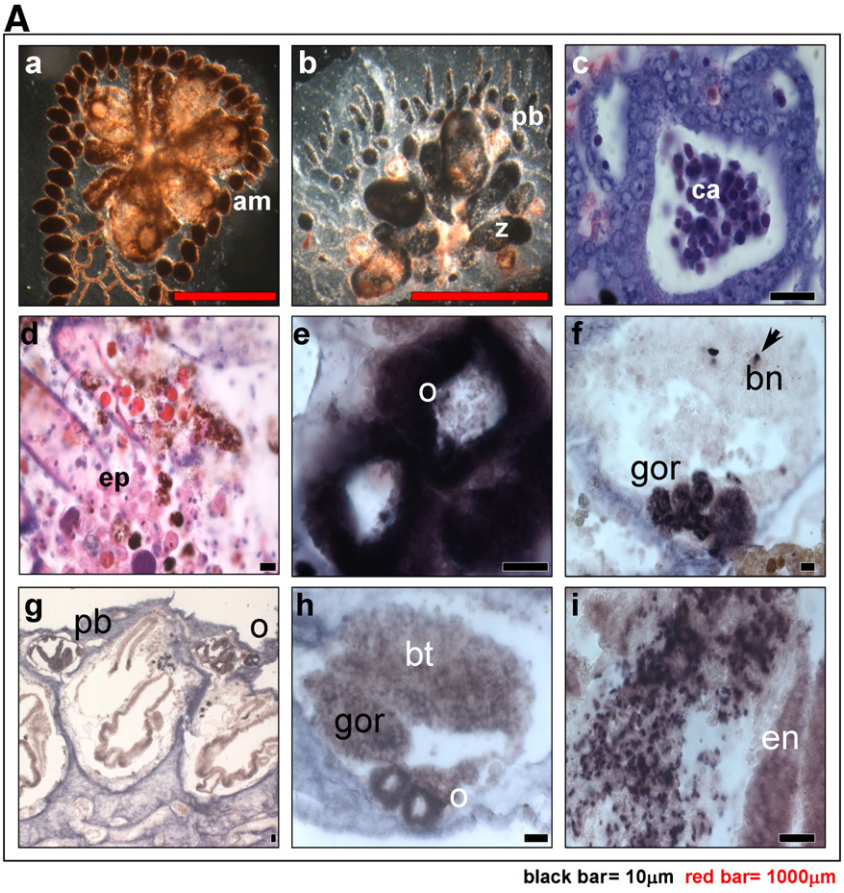

Fig. 9A. Impacts of UVB and UVC irradiation on cellular components and stem cell marker expressions. (A) Phenotypic changes in ramets irradiated by (a) UVC, or by (b) UVB were further analyzed by (c–d) Hematoxylin–eosin. (c) UVB irradiated (2 h) bud contains a new cluster of basophilic cells. (d) UVB irradiated (4 h) zooid, 24 h after irradiation, an eosinophilic population of cells proliferates in the lacunae near the endostyle. Immunohistochemical analysis of these UVB irradiated colonies were performed either with (e–f) BS-Vasa antibodies or with (g–i) BS-Pl10 antibodies. The analyzed samples were irradiated for (g) 0.5 h, (e) 1 h, (h) 4 h, or (f, i) were harvested 24 h after 4 h of irradiation. Abbreviations: am—ampulla; bt—budlet; bn—budlet niche; ca—cell aggregate; en—endostyle; ep—eosinophilic cells; gor—gonad rudiment; o—oocyte; pb—primary bud; z—zooid. Arrows point to PGC-like cells.

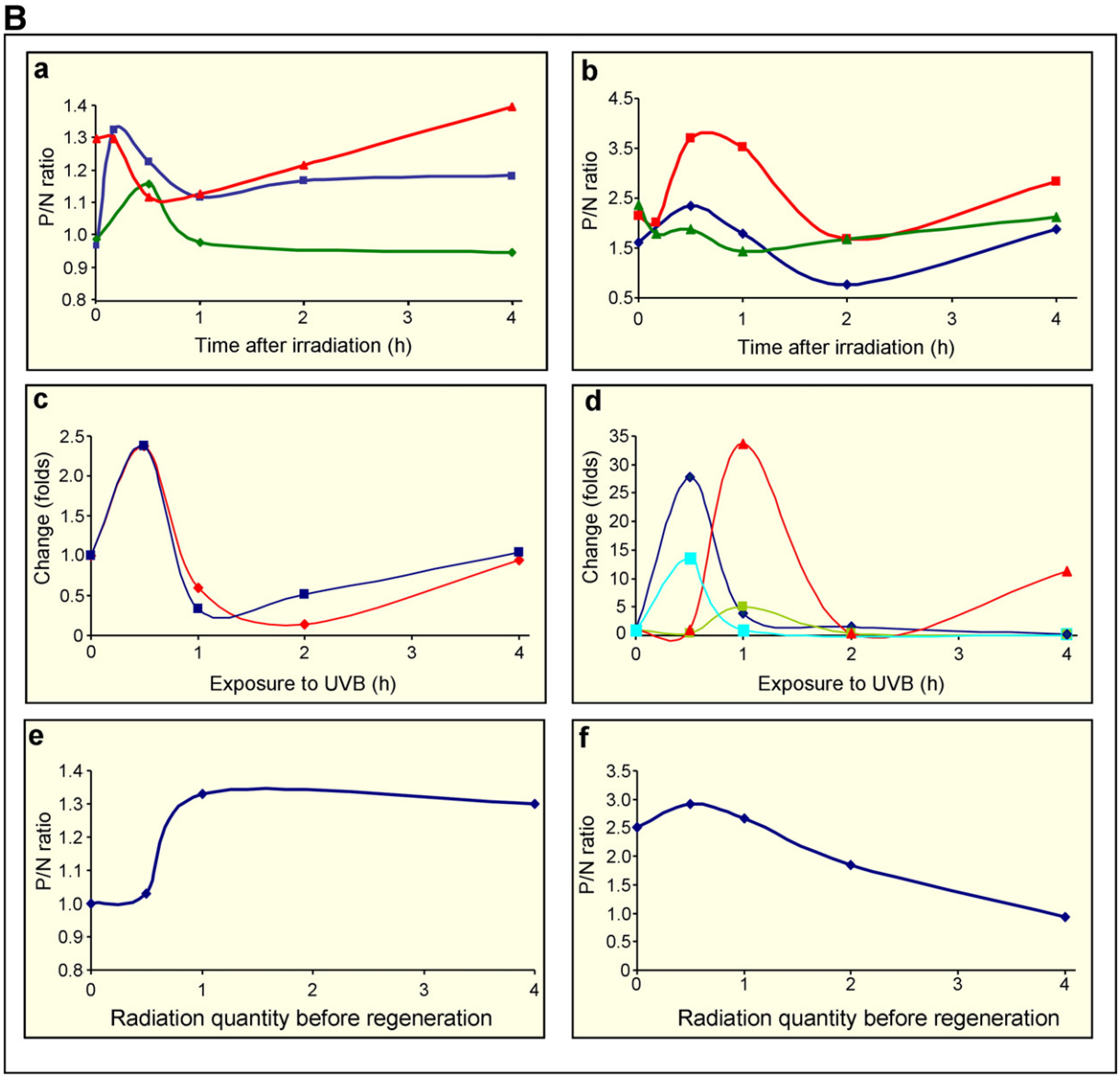

Fig. 9B. Impacts of UVB and UVC irradiation on cellular components and stem cell marker expressions. (B) Responses of BS-Vasa and BS-Pl10 mRNAs and proteins following UVB and UVC irradiation. (a) BS-Vasa and (b) BS-Pl10 proteins were quantified by ELISA as function of time after UVC irradiation. The control was an untreated ramet. The relative quantities of proteins are expressed by the ratio of the signal obtained with the specific antibody (P) divided by the signal obtained with the appropriate pre-immune serum (N). UVB irradiation effects on (c) BS-Vasa and (d) BS-Pl10 mRNA as quantified during the whole length of irradiation. mRNA quantities are expressed as folds of change relative to untreated colonies; Protein expressions of (e) BS-Vasa and (f) BS-Pl10 24 h after UVB irradiation. The horizontal axis represent the duration of UVB irradiation the ramet received before return to normal condition. Each curve represented a different genet.

species (Rosner et al., 2006; Rosner and Rinkevich, 2007), Vasa specific primers, RNA probes, and antibodies were carefully designed. Results showed that BS-Vasa gene products were strongly expressed in various cell types (Table 1) in early precursors of the germ cell lineage, as in non-germ line cells (test cells, macrophage-like cells along the endostyle, digestive system) fluctuating during blastogenesis. Three additional stem cell markers, BS-Pl10, Bl-piwi orthologue and Oct4 orthologue, were used to confirm germ lineage exclusivity (Table 1). BS-Pl10 was highly expressed in differentiating soma cells and in young staged germ cells (oocytes and testes). Oct4, marker for stem cells and PGCs (Donovan, 2001; Cheng et al., 2007), was expressed in *B. schlosseri*, also in mature oocytes, and cells in the endostyle (Fig. 3Bf), the latter has recently been identified as a stem cell niche (Voskoboynik et al., 2008). Bl-piwi, an omnipresent *bona fide* stemness flag for self-renewal and maintenance of germ line and somatic stem cells in diverse multicellular organisms (Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007; O'Donnell and Boeke, 2007), while not expressed in germ cells (implying for another piwi paralogue functioning in spermatogenesis), was found in non germ line cell populations, co-expressed with BS-Vasa (Fig. 3, Table 1).

PGC-like cells were recorded in sexual as in asexual colonies, surviving irradiation. The predetermined location of the PGC-like cells in both, asexual (as from the oozooid stage) and sexually matured organisms, marks the budlet site as a germ line niche (in parallel to the stem cell niche characterized to this group of organisms; Voskoboynik et al., 2008), which may harbors germ cells ready to populate the gonads, even in sexually pre-matured colonies.

Budding tunicates of the order Pleurogona develop new zooids through continuous cycles of blastogenesis and a unique budding process, called palleal budding (Berrill, 1950), in which all soma and gonads are *de novo* reconstructed along each blastogenic cycle (Berrill, 1940; Izzard, 1973; Mukai and Watanabe, 1976a). Studies contend that in botryllid ascidians, the gonads and gametes originate from groups of undifferentiated mesenchymal cells termed hemoblasts (Mukai and

Table 1
Summary of stem cell marker expressions in *B. schlosseri* somatic and germ cell lineages.

Cell type	Location	Marker					
		Vasa mRNA	Vasa protein	PL10 mRNA	Pl10 protein	Oct4 orthologue	Bl-piwi orthologue
PGC-like	Vasculature	+++	+++	+++	+++	+++	−
	Budlet niche	+++	+++	+++	+++	+++	−
	Gonad rudiment	+++	+++	+++	+++	+++	−
	Bud	+++	+++	+++	+++	+++	−
Oocytes	Gonad rudiment	+++	+++	+++	+++	+++	−
	Gonad sac	+++/+−	+++/+−	+++/+−	+++/+−	+++	−
Sperm	Gonad sac	++	+++	+++	+++	++	−
	Zooid	+++/−	+−	++/−	++/−	+−	−
Test cells	Mature oocytes	+++	+++	−	−	−	+++
	Young embryos	+++	+++	−	−	−	+++
	Embryo tunic	+++	+++	−	−	−	+++
Follicular cells	Mature oocytes	−	−	+++	+++	−	−
Macrophage-like	Along zooid endostyle	−	+++	+−	+++	−	+++
Epithelium	Zooidal digestive system	+/−	+++	+	+	−	+++
Embryo cells	Early stage	+−	+−	+−	+−	n/d	
	Wrap-tail	+++	+++[a]	++	+++	+++	−

n/d — not done; +++ intensive staining; ++ moderate staining; + low staining; +− very low staining; − negative staining; +++/+− staining varies from +++ to +−; ++/− staining varies from ++ to −; +/− staining varies from + to −.
[a] The staining is detected only in few cells in the middle of the body trunk.

Watanabe, 1976a; Mukai, 1977; Sabbadin and Zaniolo, 1979), which circulate freely in the coelomic spaces and in the vasculature. Those hemoblasts aggregate upon unknown signals and ultimately generate the germ cells and follicular cells of the gonads (Sunanaga et al., 2006) by fast cell division cycles (Kawamura et al., 2008). This model is backed by experimental chimeras established between immunological competent genotypes that demonstrate full or partial replacement of germ or somatic cells (germ or somatic cell lineage parasitism) of one of the genotypes by cells of the other (Sabbadin and Zaniolo, 1979; Pancer et al., 1995; Stoner et al., 1999; Rinkevich, 2002). By transplanting limiting dilution series of donor blood cells, Laird et al. (2005) suggested that somatic stem or germ cells reside and proliferate in explicit compartments and that stem cells found in the blood are those, which migrate via blood to buds or gonads. Here, we identified, for the first time, these putative compartments in asexual and sexual animals.

Whereas preformistic mode of development (according to Extavour and Akam approach) was assigned to the solitary tunicate (Shirae-Kurabayashi et al., 2006), *de novo* epigenetically-like differentiation of hemoblasts into female germ cells was described in the colonial tunicates *B. primigenus* and *P. misakiensis* (Sunanaga et al., 2006, 2007). In *B. schlosseri*, the prevailing dogma is that germ and somatic stem cell lineages separate (Stoner et al., 1999) at early stages of ontogeny when the germ cell lineage is set aside to follow the unique pathway of differentiation into gametes. This hypothesis is supported by Laird et al. (2005), showing that transplantation of a low number of donor cells between *B. schlosseri* genotypes may result in either germ or soma descendants in the recipient colony and by the present study characterization of $Vasa^+$, $Oct4^+$ and $Pl10^+$ PGC-like cells population in the larvae and oozooids. This population might be the origin of all germ cells in the colony. The alternative scenario advocates for a single pool of totipotent cells along ontogeny that is set aside, at any ontogenic window time, and generates germ and soma derivatives (somatic embryogenesis mode of development). This is supported by findings that *Vasa* positive cells, whereas detected from the larva and the oozooid stages, are repeatedly appearing in the colony, independently of its sexual state, emerging also in cell lineages not related to the germ line.

The existence of non-clustered Vasa positive PGC-like cells in *B. schlosseri* is in contrast to the observations in the closely related colonial ascidians *B. primigenus* and *P. misakiensis* (Sunanaga et al., 2006, 2007). This difference might reflect different modes of germ lineage sequestering between the species or might pose a doubt on Vasa mRNA as reliable and sensitive molecular marker that can trace germ line-committed precursors. In the latter case, it could be that either non-clustered germ cell precursors do exist, but they do not express Vasa genes products, alternatively they express only Vasa protein.

Following the characterization of the first *Vasa* gene, which was originally identified in *Drosophila* as a maternal-effect gene required for germ cell specification (Schupbach and Wieschaus, 1986), numerous studies have illuminated the possibility that Vasa is not restricted to the germ cell lineage and is expressed in totipotent, multipotent, or even in specific differentiated soma cells. Vasa orthologues are expressed in the soma of planarians, in neoblasts (Shibata et al., 1999; Pfister et al., 2008), whereas in cnidarians (i.e., *Hydra*, *Hydractinia*, *Nematostella*) Vasa is expressed throughout the entire life cycle in multipotent stem cells called interstitial stem cells (Mochizuki et al., 2001; Extavour et al., 2005; Rebscher et al., 2008). Vasa is expressed in the stem cells of the mesodermal growth zone in the polychaete *Platynereis* (Rebscher et al., 2007; Dill and Seaver, 2008). In the sea urchin, Vasa functions also in an early stem cell population of the embryo (Voronina et al., 2008). It is expressed in the soma of the parasitic rhizocephalan barnacles (Shukalyuk et al., 2007) and in somatic cells as well as in germ line cells of *Xenopus* (Ikenishi and Tanaka, 2000). Here, we also showed that BS-Vasa gene products are not exclusively expressed in germ lineages. BS-Vasa mRNA is expressed in many embryonic and bud somatic cells (Fig. 5C) while BS-Vasa protein is expressed in various soma cell types. These include test cells, which wrap the oocytes and the embryos (Zaniolo et al., 1989), the macrophage-like cells along the endostyle of adult zooids (not stained by the BS-Vasa mRNA), and cells of the stomach (Table 1). Of these three cell populations, the most intriguing are the test cells (which do not express PL10 or Oct4) because they are maternally derived and are transferred to the embryos, where they are found in the tunic. Various maintenance functions that are assigned to these cells in the literature (Sato et al., 1997) do not explain BS-Vasa gene products and Bl-piwi expressions, although it is clear that they are somatically differentiated. The staining of the macrophage-like cells and of the stomach cells, on the other hand, were not definitely excluded from being unspecific (Supplementary section), and therefore they should be handled with precaution.

In contrast to its co-expression with Pl10 and Oct4 in germ line (Table 1), the co-expression of BS-Vasa with piwi in somatic cells

could indicate that BS-Vasa has additional functions to the germ line assignments (a suggestion supported by the BS-Vasa response to UV irradiation; Vashisht and Tuteja, 2006), or that *B. schlosseri* reveals a somatic embryogenesis mode of development. This confronts the prevailing dogma for epigenetic mode of development. However, both alternatives are not necessarily mutually exclusive. Additional studies are needed to further elucidate PGC fate, mode of ontogenic development and full repertoire of BS-Vasa functions in this group of colonial organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.04.025.

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