Developmental Biology 384 (2013) 356–374

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Evolution of Developmental Control Mechanisms

Germ lineage properties in the urochordate *Botryllus schlosseri* – From markers to temporal niches

Amalia Rosner*, Elizabeth Moiseeva, Claudette Rabinowitz, Baruch Rinkevich

National Institute of Oceanography, Israel Oceanography & Limnological Research, Tel Shikmona, P.O. Box 8030, Haifa 31080, Israel

ARTICLE INFO

Article history:
Received 21 September 2012
Received in revised form
25 August 2013
Accepted 3 October 2013
Available online 9 October 2013

Keywords:
Botryllus schlosseri
BS-cadherin
BS-DDX1
BS-Vasa
γ-H2AX
Germ cells
Niche
PGC
Phospho-Smad1/5/8
RNA helicase
Urochordata

ABSTRACT

The primordial germ cells (PGCs) in the colonial urochordate *Botryllus schlosseri* are sequestered in late embryonic stage. PGC-like populations, located at any blastogenic stage in specific niches, inside modules with curtailed lifespan, survive throughout the life of the colony by repeated weekly migration to newly formed buds. This cyclical migration and the lack of specific markers for PGC-like populations are obstacles to the study on PGCs. For that purpose, we isolated the *Botryllus* DDX1 (BS-DDX1) and characterized it by normal expression patterns and by specific siRNA knockdown experiments. Expression of BS-DDX1 concurrent with BS-Vasa, γ-H2AX, BS-cadherin and phospho-Smad1/5/8, demarcate PGC cells from soma cells and from more differentiated germ cells lineages, which enabled the detection of additional putative transient niches in zooids. Employing BS-cadherin siRNA knockdown, retinoic acid (RA) administration or β-estradiol administration affirmed the BS-Vasa$^+$BS-DDX1$^+$BS-cadherin$^+$γ-H2AX$^+$phospho-Smad1/5/8$^+$ population as the *B. schlosseri* PGC-like cells. By striving to understand the PGC-like cells trafficking between transient niches along blastogenic cycles, CM-DiI-stained PGC-like enriched populations from late blastogenic stage D zooids were injected into genetically matched colonial ramets at blastogenic stages A or C and their fates were observed for 9 days. Based on the accumulated data, we conceived a novel network of several transient and short lived ‘germ line niches’ that preserve PGCs homeostasis, protecting these cells from the weekly astogenic senescence processes, thus enabling the survival of the PGCs throughout the organism's life.

Introduction

A colony of the urochordate *Botryllus schlosseri*, the descendent of a sexually reproduced swimming larva, is a hermaphroditic organism (reviewed in Berrill, 1940, 1950, 1951; Satoh, 1994) with male and female germ cells deriving from circulatory cells in the colonial vasculature. Each colony is composed of a few to several thousand genetically identical modules (formed through body wall evagination) belonging to three successive asexual generations, the functionally filtering zooids and two cohorts of palleal primary buds (1–4 per zooid) and budlets (secondary buds). All modules of a particular generation are synchronized to be at exactly the same developmental phase. Functional zooids are repeatedly replaced by primary buds on a weekly basis (under 18–20 °C), in a process called blastogenesis (divided into four major stages A–D; sensu Mukai and Watanabe, 1976) through pan-colonial coordinated apoptotic and phagocytosis waves, concurrent with the fast development of primary buds into the adult zooid stage (blastogenic stage D, also called the ‘takeover’ phase). All tissues and organs, including the gonads, are reconstructed during every single blastogenic cycle (Berrill, 1940; Izzard, 1973). While blastogenesis starts from the first week of larval metamorphosis and continues throughout the colony's lifespan, sexual reproduction ensues several sterile blastogenic cycles with the formation of testes in buds. Sporadic formation of egg follicles within buds, dorsally to the testes, may complement sexual maturity several blastogenic cycles later. Ovulation takes place within the newly formed zooids, simultaneously with the opening of the siphons and the beginning of a new blastogenic cycle, while sperm matures within the testes situated in the functional zooid (Milkman, 1967; Mukai, 1977; Rinkevich, 2002).

One of the most intriguing questions is the origin of gametes in colonial organisms. The potential precursors for germ stem cells (GSC) in ascidians, which were studied in several species (Kawamura et al., 2011), including the compound ascidians *Botryllus primigenus* (Sunanaga et al., 2006, 2010; Kawamura and Sunanaga, 2011), *B. schlosseri* (Brown et al., 2009; Rosner et al., 2009) and *Polyandrocarpa misakiensis* (Sunanaga et al., 2007), failed to produce a common paradigm. To elucidate the molecular mechanisms underlying germ line sequestration and the identification of germ cell precursors in *B. schlosseri*, we first used the

---

* Corresponding author. Fax: +972 4 8511911.
E-mail addresses: amalia@ocean.org.il, buki@ocean.org.il, amalia.rosner091@gmail.com (A. Rosner).

0012-1606/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2013.10.002

classical germ line marker BS-Vasa (Rosner et al., 2009) to illuminate a group (6–12 μm size) of BS-Vasa$^+$ cells assumed to represent the Primordial Germ Cells (PGC-like, Fig. 1a1) already seen in embryos. Following larval metamorphosis and the establishment of young colonies, clustered BS-Vasa$^+$ cells were found attached to the epithelia in regions where, in gravid colonies, nascent and mature gonads are typically formed (Rosner et al., 2009). In sexually reproducing colonies, these cells were situated within two budlet niches (termed as the 'budlet niche' and 'gonad rudiment' Fig. 1a2 and a3) and within the 'gonad sacs', or sporadically attached to soma tissues of primary buds (Fig. 1a3). In view of the aforementioned experiments and additional observations, we defined the *B. schlosseri* PGC-like population as 'BS-Vasa$^+$ Oct 4$^+$ Pl10$^+$' cells, easily highlighted by this battery of markers from bud soma tissue. These PGC-like cells, which were marked by particular Vasa$^+$ granules arranged around the nucleus (as in PGCs of other organisms; Strasser et al., 2008) were the first cell type to be observed in regions in which gonads would eventually be formed.

Recently, Rinkevich et al. (2013) have identified an additional germ niche situated in the zooidal cell islands (CI) along the endostyle and demonstrated that cells originating from these islands migrate to the buds and are capable of differentiating into germ cells. The migrating cells (Voskoboynik et al. 2008; Lauzon et al., 2013; Rinkevich et al., 2013) include several cell populations: GSC, soma stem cells and phagocytes, all marked by BS-Vasa$^+$ Oct 4$^+$ Pl10$^+$ (Rosner et al., 2006, 2009; Rosner and Rinkevich., 2011), making it difficult to compare between the various germ stem cell populations. New markers that typify different germ cell populations are therefore a necessary tool for the study of germ line sequestering.

Migration of PGCs from their site of specification towards the developing gonads which was studied in many animal models (Dudley et al., 2010) revealed the strict control during this journey aiming at the preserving PGCs 'stemness identity' (Gu et al., 2009). Once settled in developing gonads, PGCs stemness identity continues to be preserved in fixed specific niches throughout the lifespan of the organism (Murray et al., 2010). In *B. schlosseri*, where zooids have curtailed longevity and germ stem cell long-term stockpiling is mediated via recycle between mature (zooids) and developing (buds) tissues, effective characterization and tracing of PGC cells may uncover new mechanisms, which enable the preservation of the 'stemness identity'.

In looking for additional markers of germ line precursors, we targeted DDX1, a gene commonly enriched in mouse PGC (Tanaka et al., 2009). Like Vasa, DDX1 is an RNA helicase (reviewed in Linder and Jankowsky, 2011; Pyle, 2011) ubiquitously found in unicellular (e.g., *Dictyostelium discoideum*) and in all the currently studied animals (*Nematostella vectensis* to humans). Activity of this molecule is associated with numerous biochemical functions related to RNA metabolism, such as regulation of transcription (Ishaq et al., 2009), translation and signal transduction (Chen et al., 2002), spliceosome assembly (Rzymski et al., 2008), alternative splicing regulation (Ho et al., 2004; Pascual et al., 2006; Onishi et al., 2008; Vicente-Crespo et al., 2008), polyadenylation (Bléoo et al., 2001; Chen et al., 2002), RNA transport (Kanai et al., 2004), cell cycle control (Tanaka et al., 2009), DNA double-strand breaks repair (DSBs, Li et al., 2008), stress (Buchan and Parker, 2009), immunity (Zhang et al., 2011) and oogenesis (Rafti et al., 1996). Associated with its wide range of biological functions, DDX1 is also implicated in cancers (Godbout and Squire, 1993; Squire et al., 1995; Tanaka et al., 2009; Balko and Arteaga, 2011; Taunk et al., 2011) and in certain viral infections (Fang et al., 2004, 2005; Tingting et al., 2006; Sunden et al., 2007a, 2007b).

This study confirms BS-DDX1 expression in cytoplasm of PGC-like, male and female gonia cells, and in combination with other markers (BS-Vasa, BS-cadherin, γ-H2AX, and phospho-Smad1/5/8) typifies the PGC-like populations. BS-cadherin knockdown assays unveil the significant function of BS-cadherin in PGC clustering phenotype and development. Alteration of retinoic acid (RA) levels, administration of β-estradiol and injections of labeled PGC-like enriched populations, further revealed the nature of these cell populations, helping in the identification of new PGC niches and the cells traveling between niches during the blastogenic cycle.

Experimental procedures

Animals

B. schlosseri colonies collected at Monterey, Half Moon Bay and Moss Landing Marinas, California, were reared at 20 °C as described by Rinkevich and Shapira (1998).

Isolation of BS-DDX1 cDNA

A partial sequence of the DDX1 gene was obtained from an EST library available in our laboratory. Regions 5′ and the 3′ of this fragment were obtained using 'SMART RACE cDNA Amplification Kit' (Clontech, CA, USA) with the following changes: 3′ RT was performed with RevertAid™ Premium Reverse Transcriptase (Fermentas Life sciences, www.fermentas.com). 5′ RT was performed with Takara Bio's PrimeScript™ reverse Transcriptase.

In situ mRNA hybridization

Probes were labeled and hybridization was performed as per Rosner et al. (2009). Hybridization and washings were performed at 60 °C. Two DDX1 specific probes were used (Supplementary Fig. 1): (I) a 229 bp fragment ranging nucleotides 1138–1367 of the cDNA or amino acids 367–443 of the predicted protein and (II) a 360 bp fragment ranging nucleotides 1525–1884 of the cDNA or amino acids 496–615 of the predicted protein.

Primary antibodies

Anti-BS-DDX1 antibodies were produced by injecting the peptide CSATLHSFDVKKLAEKIMRF conjugated to chick albumin into rats or rabbits. Rat serum was further purified and used at dilution of 1:150. The rabbit serum was used at dilution of 1:4000. Rabbit anti-phospho-Smad1/Smad5/Smad8 #9511 produced by Cell Signaling (www.cellsignal.com) used at dilution of 1:100. Rabbit anti-phospho-Smad2 #3101 Cell Signaling (www.cellsignal.com) used at dilution of 1:200. Commercial rabbit anti-gamma H2A.X #ab11174 produced by Abcam (Cambridge UK) used at 1:1000 concentration. Rabbit anti-BS-cadherin, Rabbit anti-BS-Vasa were previously elicited in our laboratory as described (Rosner et al., 2007, 2009).

Secondary antibodies

Alkaline Phosphatase-conjugated AffiniPure Donkey anti-Rat IgG (H+L) at dilution 1:10,000 (Cat. no. 712-055-153; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Cy™-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) Amax-550; Emax 570 (Cat No. 111-165-003; Jackson ImmunoResearch Laboratories). Alkaline Phosphatase-conjugated Goat anti-Rabbit IgM or IgG were purchased from Southern Biotech (Cat. no. 4020-04; Birmingham AL, USA) or Jackson ImmunoResearch (Cat. no. 111-055-