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Regeneration of the germline in the annelid *Capitella teleta*

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ABSTRACT

The germline is essential for sexual reproduction and survival of the species. In many metazoans, the developmental potential to generate a distinct germline is segregated from somatic cell lineages early in embryogenesis, suggesting that the unique features of the germline must be established from its onset. Previous studies suggest that germ cells cannot regenerate once removed from the embryo, but few animals have been experimentally tested. We investigated the ability of the germline to regenerate in a lophotrochozoan, the segmented worm *Capitella teleta*, which has a stereotyped cell lineage program by deleting the germline precursor (cell 3D) in early stage embryos using an infrared laser. Larvae and juveniles resulting from germline deletions were examined for presence of multipotent progenitor cells (MPCs), stem cells that form the germ cells and somatic stem cells. In contrast to control deletions of a non-germline macromere, most larvae resulting from deletion of cell 3D lacked MPCs as assayed by expression of germline markers *CapI-vasa*, *CapI-nanos* and *Ct-piwi1*, but showed persistent expression of these markers in the somatic posterior growth zone. However, approximately 13% of experimental larvae had MPCs, indicative of some germline regeneration. In contrast, by two weeks post-metamorphosis, all juveniles resulting from deletion of cell 3D had MPCs, as detected by *CapI-vasa* expression. Furthermore, when raised to adulthood, most animals developed reproductive structures and were fertile. In another set of deletions, both the D quadrant mesodermal and germline progenitors were removed. These juveniles also regenerated MPCs. Surprisingly, this deletion caused substantial ectopic expression of *CapI-vasa* and *CapI-nanos* in other larval tissues. Our results indicate that *C. teleta* can regenerate the germline following removal of the germline progenitors in the early embryo. The dramatic difference in ability to regenerate the germline between the larval and adult stages suggests that there are two distinct compensation events at two phases of the life cycle: a regulative event in the early stage larva and a stem cell transition event after metamorphosis, when the animals are capable of substantial body regeneration.

1. Introduction

The germline is necessary for sexual reproduction, which is imperative for the survival and evolution of species. In many well-studied bilaterian organisms, the germline separates completely from the somatic cells early in embryonic development. This can occur either by sequestration of proteins and mRNA in the cytoplasm of the zygote, or by induction via a cell-signaling event from other cells in the embryo. These two distinct mechanisms are known as preformation and epigenesis, respectively (Nieuwkoop and Sutasurya, 1979, 1981; Extavour and Akam, 2003). The segregated cells that will later form the sperm and egg are known as primordial germ cells (PGCs). It has been proposed that in many animals, germline and somatic lineages must separate early in embryonic development to avoid evolutionarily detrimental competition between different cell lineages within the organism (Buss, 1987). In addition, a number of studies have shown that the germline undergoes transcriptional and translational silencing to minimize the possibility of passing on somatic mutations to the germline, and these characteristics are not shared with somatic lineages (Blackler, 1970; Drake et al., 1998; Milholland et al., 2017; Seydoux and Braun, 2006; Strome and Updike, 2015; reviewed in Weisblat, 2006).

Removal of germ cells by excision, irradiation, or deletion results in sterile adults in many organisms, including in *Mus musculus*, *Xenopus laevis*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Ambystoma mexicanum*, and *Gallus gallus* (Barnes et al., 2006; Blackler, 1965; Buehr and Blackler, 1970; Dubois, 1962; Dulbecco, 1946; Everett, 1943; Fargeix, 1975; Nieuwkoop, 1951; Reynaud, 1976; Sulston and Schierenberg, 1983; Züst and Dixon, 1975). These animals develop reproductive structures with no gametes. The results of such experimental manipulations support the idea of complete separation between the germline and soma early in the developmental program.

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Once segregated, the germline cannot reform from other cell types, demonstrating a single embryonic origin of the germline. In contrast, more recent experiments in the ascidian *Ciona intestinalis* have uncovered an example of germline regeneration. In *C. intestinalis*, the germline precursors are located in the tail during the larval tadpole stage, and when the larval tail is removed, the resulting juveniles lack germ cells. However, after 15 days, a few germ cells appear, and later, the adults produce sperm (Takamura et al., 2002). It is unknown from where these cells arise and if this example is a rare occurrence or whether additional sampling will reveal more cases of animals that can regenerate their germline. It is worth noting that experimental manipulations of the germline have only been performed on a small fraction of animal clades.

The superphylum Lophotrochozoa contains 14 or so highly diverse animal phyla and relative to other clades, little is known about the development of the germline, and whether its members have an ability to regenerate their germline (reviewed in Extavour and Akam, 2003). Many members of this superphylum have a shared developmental program called spiral development. Embryos that undergo spiral cleavage have a stereotypic cleavage pattern such that each cell within the embryo can be identified based on spatial relationships, cell size, and time of division (Wilson, 1892; Henry and Martindale, 1999, 1998). The predictable cleavage pattern of spiralian embryos has enabled researchers to perform single cell blastomere fate map and deletion studies on several species (Ackermann et al., 2005; Boyer et al., 1996; Damen and Dictus, 1994; Hejnol et al., 2007; Henry and Martindale, 1998; Maslakova et al., 2004; Meyer et al., 2010; Render and Render, 1997; Weisblat and Shankland, 1985). From these studies, it has been shown that several aspects of the fate map are conserved across species and even across phyla. One notable example is the apparent conservation of the embryonic origin of the germline. In all animals examined, the precursor of the germline is localized to a single cell in the 64-cell stage embryo, the cell 4d (reviewed in Lambert, 2008).

One lophotrochozoan, the annelid *C. teleta,* has several advantages for studies of the germline. The embryonic origin of the germline in *C. teleta* appears to be conserved with that of other spiralians. Cell lineage and gene expression studies using the genes *piwi*, *nanos* and *vasa* are consistent with the idea that descendants of cell 4d coincide with germline precursor stem cells (Dill and Seaver, 2008; Giani et al., 2011; Meyer et al., 2010). However, unlike in other spiralians, the germline and the mesoderm do not arise from the same precursor cell, allowing for experimental manipulation of the germline without also disrupting mesoderm formation (Meyer et al., 2010). In addition, *C. teleta* reproduces sexually with separate male and female sexes that can be successfully mated in the laboratory. Adult reproductive structures are morphologically visible and have previously been characterized in detail (Eckelbarger et al., 1984; Eckelbarger and Grassle, 1987a, 1987b). Furthermore, similar to many other annelids, *C. teleta* can regenerate (Bely, 2006; Bely et al., 2014). For example, following transverse amputation, *C. teleta* can regenerate its nervous system, musculature, and digestive tract (de Jong and Seaver, 2016). Both somatic (ovaries) and germline (oocytes) components of the reproductive tissues can also regenerate following amputation posterior of the 6th thoracic segment (Giani et al., 2011; Hill and Savage, 2009). The ability of *C. teleta* to regenerate multiple tissue types led us to hypothesize that this animal may have a unique stem cell regulatory program that allows transition of somatic stem cells to germ cells, and to potentially regenerate the germline.

Historically, morphological similarities have been observed between germ cells and stem cells in annelids (Faulkner, 1932; Potswald, 1972, 1969). Notably, both cell types have a large nuclear to cytoplasmic ratio and a characteristic morphology of undifferentiated cells. More recent molecular studies, including studies in *C. teleta,* show that the markers *vasa*, *nanos*, and *piwi* are expressed in both the germline and somatic stem cell populations in many species, emphasizing the similarities between the germline and stem cells (Dill and Seaver, 2008; Fischer and Arendt, 2013; Giani et al., 2011; Lyons et al., 2012; Mochizuki et al., 2001; Özpolat et al., 2016; Raz, 2002; Rebscher, 2014; Rebscher et al., 2012; Shibata et al., 1999; Solana, 2013). Such genes are proposed to have a role in maintaining an undifferentiated state (Mochizuki et al., 2001). These cells have been referred to as 'germline cell stem cells', 'germinal cells', 'pre-primordial germ cells (pre-PGCs)', 'presumptive primordial germ cells (PGCs)' or 'primordial stem cells (PriSCs)' and 'molecular progenitor cells' depending upon the study. In previous studies, we referred to a cluster of cells with these characteristics in *C. teleta* cells as presumptive primordial germ cells (Giani et al., 2011), and we now refer to them as the multipotent progenitor cell (MPC) cluster to better represent additional roles that the cells in this cluster appear to have during regeneration (de Jong and Seaver, 2017). The MPC cluster in *C. teleta* larvae, juveniles, and adults can be visualized with the germline/stem cell markers *CapI-vasa*, *Ct-piwi1, Ct-piwi2,* and *CapI-nanos* (Dill and Seaver, 2008; Giani et al., 2011). These cells are either pluripotent stem cells capable of forming both germline and somatic cells, or a mixed population of cells with distinct subsets destined to become either germline or somatic stem cells. We favor the latter possibility, due to recent molecular evidence suggesting heterogeneity of cells within the cluster. Specifically, only a small subset of cells in the MPC cluster expresses the marker *Ct-myc* (de Jong and Seaver, 2017).

To determine whether *C. teleta* can regenerate its germline, we performed single cell laser deletion experiments to remove the germline precursor cell and examine the resulting effects in larvae, juveniles and adults. We assessed MPC presence using molecular markers in larvae and juveniles. Adults resulting from embryonic deletion of the germline precursors were analyzed for the presence of reproductive structures, ability to mate, and viability of their offspring. In addition, we also investigated the cellular origin of the lineage capable of replacing the germline. To our knowledge, this work provides one of only a few examples of germline regeneration in bilaterian animals.

2. Materials and methods

2.1. Animal care

Embryos were acquired by separating gravid males from females for three to six days, and then combining them together in a mating dish for 11–14 h. Dishes were inspected for the presence of brood tubes made by the females (Seaver et al., 2005), and embryos were dissected from the brood tubes and placed in a dish of 0.2 μm-filtered seawater (FSW). All embryos and larval stages were raised in FSW with 60 μg/mL penicillin (Sigma-Aldrich) and 50 μg/mL streptomycin (Sigma-Aldrich) at 19 °C, which was exchanged each day until larval day nine. Animals were staged according to a published staging chart (Seaver et al., 2005). All juvenile and adult animals were maintained in organically enriched mud.

2.2. Cell deletions

Single blastomere deletions were performed using the XYClone system infrared laser (Hamilton Thorne) with the 20× objective fitted to a Zeiss Axioplan compound microscope as described in Yamaguchi et al. (2016). Embryos were placed on a Rainex-coated slide in a drop of FSW and oriented with the vegetal side of the embryo facing up. A chamber to cover the embryos was made by attaching two cover slip slivers on each end of a coverslip with melted dental wax following Lyons et al. (2012). For all blastomere deletions, the laser power was set to 100%, and the pulse length was adjusted based on the sensitivity of the brood, the size of the cell being targeted, and the stage of the cell cycle. The pulse range for cell 3D and 3B was one pulse between 150 and 250 μs, followed by a second pulse of 550–750 μs. Cell 2D and cell 2C were deleted using two pulses between 350 and 450 μs each. The