Preface

Stem cells are responsible for maintaining tissue homeostasis. They possess unlimited self-renewal potency. Through asymmetric cell division, a stem cell in adult tissues can produce an offspring that will maintain the stem cell populations and a daughter cell that will differentiate into various short-lived cell types to replace damaged or dying cells. Tumors may originate from a few transformed cancer stem cells. Stem cells have immense potential for therapeutic use in regenerative medicine and for developing anticancer therapies that specifically eliminate cancer stem cells. Germline stem cells are the source of human and animal reproduction. The knowledge gained from studying germline stem cells may find immediate application in preserving endangered wildlife, managing commercial livestock, overcoming fertility problems in humans, and treating testicular and ovary tumors.

To make use of this potential, we have to learn how to isolate, characterize, and maintain germline stem cells. To isolate, characterize, and maintain stem cells, we must first understand the molecular parameters that define a germline stem cell and the mechanisms that regulate stem cell behavior. The protocols in *Germline Stem Cells* are intended to present selected genetic, molecular, and cellular techniques used in germline stem cell research. The book is divided into two parts. Part I covers germline stem cell identification and regulation in model organisms. Part II covers current techniques used in in vitro culture and applications of germline stem cells. Each chapter begins with a brief overview of the topic, list of necessary equipment and reagents, step-by-step laboratory protocols, and tips on trouble-shooting and avoiding known pitfalls. We hope that *Germline Stem Cell* protocols provides basic techniques to cell and molecular biologists, tissue engineers, clinicians, geneticists, and students involved in various aspects of germline stem cell research and application.

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Chapter 2
Analysis of the *C. elegans* Germline Stem Cell Region

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Contents

2.1 Introduction 27
2.2 Materials 29
2.3 Methods 30
2.4 Notes 40
References 42

Summary We present methods for characterizing the mitotic and early meiotic regions of the *Caenorhabditis elegans* germline. The methods include examination of germlines in living and fixed worms, cell cycle analysis, analysis of markers, and initial characterization of mutants that affect germline proliferation.

Keywords BrdU; *C. elegans*; cell cycle; germline; meiosis; mitosis; proliferation; stem cells.

2.1 Introduction

Identification of stem cells and the pathways that regulate them is important for both clinical research and more basic biomedical science. The *Caenorhabditis elegans* germline is a simple and well-studied model for understanding the genetic and molecular regulation of stem cells ***(1–3)***. First, all stages of germ cell development, from stem cell to differentiated gamete, are present in the adult gonad at one time (*see* **Fig. 2.1A**). Second, establishment and maintenance of *C. elegans* germline stem cells (GSCs) is controlled by a single somatic cell, the distal tip cell (DTC) (*see* **Fig. 2.1A**), which provides the niche for the stem cells ***(4)***. Finally, regulators of stem cell self-renewal have been identified and analyzed in depth (*see* **Fig. 2.1B**). Both the Notch signaling pathway ***(5)*** and the PUF (for Pumilio and FBF) family of RNA regulators maintain stem cells in *C. elegans* ***(3,5)***. These regulators are also likely to control stem cells in other systems. For example, Notch signaling has been suggested to play roles in stem cell regulation in vertebrates (for review, *see* **ref. *6***).

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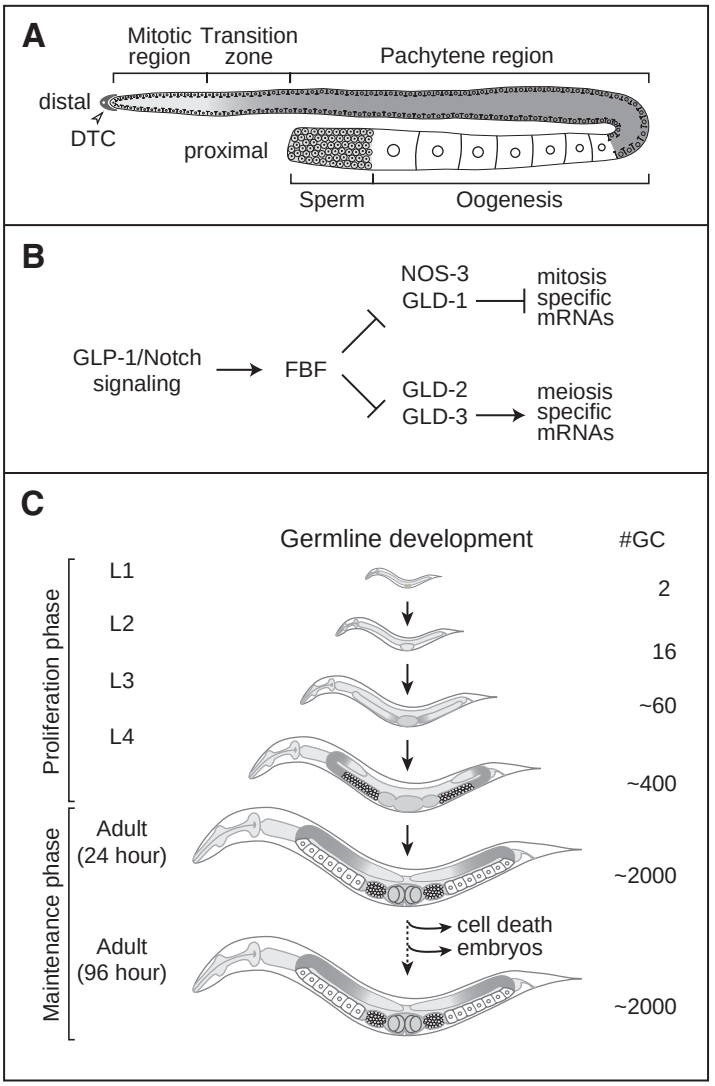

Fig. 2.1 Introduction to *C. elegans* germline development. **A** Diagram of one arm of the adult hermaphrodite germline. The somatic distal tip cell (DTC), *arrowhead*, is located at the distal end and provides a niche for proliferating germline stem cells. Germ cells differentiate as they move away from the DTC, toward the proximal end of the germline. **B** Pathway controlling the proliferation/differentiation switch in the *C. elegans* germline. **C** Diagram showing the stages of germline development from the larval proliferative phases through the adult maintenance phase. Approximate germ cell numbers for each stage are given on the *right*

PUF proteins are required for GSC self-renewal in flies (*see* **ref.** ***7*** and references therein) and have been found in human spermatogonia and ES cells ***(8)***.

We describe methods for studying *C. elegans* GSCs. Criteria to identify stem cells can vary; thus, it is crucial to define what is known in the system under consideration and to define the criteria for identifying cell types. In the *C. elegans* germline, mitotic cells are at the distal end, adjacent to the DTC (*see* **Fig. 2.1A**). The mitotic cells self-renew and contribute to the more proximal meiotic population, replenishing the germline as mature gametes are lost (*see* **Fig. 2.1A**). The GSCs reside within the mitotic region of the germline; however, their position and numbers have not been unambiguously identified. A subset of mitotic germ cells in the few rows closest to the DTC expresses high levels of the mitotic activators GLP-1, FBF-1, and FBF-2 and low levels of the meiotic activator GLD-1 (*see* **Figs. 2.1B** and **2.6**) ***(3,5)***. Because of their expression pattern and their position near the DTC, this subset is likely to include the GSCs. Mitotic germ cells further from the DTC begin to express markers of differentiation, such as GLD-1 and HIM-3 ***(9–11)***. These germ cells may be analogous to transit-amplifying cells in other systems. Based on analyses of mitotic index, S-phase index, and molecular markers, some of these more proximal cells appear to be in premeiotic S-phase ***(10,12)***. All mitotic germ cells, including stem cells, cycle at similar rates and divide with a random orientation ***(12–14)***.

We present methods for identifying and characterizing undifferentiated and proliferating germ cells, including identification of the proliferative region, premeiotic S-phase region and putative stem cells. We then discuss how we characterize new mutants using procedures for wild-type germlines. There are excellent chapters about other useful techniques freely available on the WormBook web site (http://www.wormbook.org) (*see* **Subheading 2.2**).

2.2 Materials

2.2.1 Reagents

1. 4% agarose in distilled water (dH_2O) for microscopy of live *C. elegans*.
2. M9: 22 m*M* KH_2PO_4, 22 m*M* Na_2HPO_4, 85 m*M* NaCl, 1 m*M* $MgSO_4$.
3. M9 plus 0.25 m*M* levamisole.
4. Slides and coverslips.
5. Subbing solution: Bring 200 mL dH_2O to 60°C, then add 0.4 g gelatin; cool to 40°C. Add 0.04 g chrome alum and sodium azide to 1 m*M*. Add poly-L-lysine (Sigma, cat. no. P1524) to 1 mg/mL. Store subbing solution at 4°C. To sub slides, put subbing solution on slide for 10 min at room temperature. Wick off excess liquid. Dry in 65°C oven for approx. 30 min. Slides can be stored in the oven or at room temperature.
6. Paraformaldehyde: 16% stock (Electron Microscopy Sciences, cat. no. 15710).
7. PBSB: Phosphate-buffered saline (PBS; for 1 L: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH to 7.2 with NaOH) containing 0.5% bovine serum albumin (BSA).

8. DAPI (4′,6-diamidino-2-phenylindole) (Molecular Probes, Invitrogen cat. no. D1306).
9. Vectashield (Vector Labs, cat. no. H-1000).
10. Hoechst 33342 (Molecular Probes, Invitrogen cat. no. H3570).
11. SYTO-12 (Molecular Probes, Invitrogen cat. no. S7574).
12. Rabbit antiphosphohistone H3 (PH3) polyclonal antibody (Upstate Biotechnology, cat. no. 06–570).
13. Mouse anti-PH3 monoclonal antibody (Cell Signaling Technology, cat. no. 97065).
14. M9–agar plates: 1.2% agar, 0.6% agarose in M9 salts containing 0.1 mg/mL ampicillin ***(15)***.
15. BrdU (bromodeoxyuridine) (BD-Pharmingen, cat. no. 550891). Store aliquots at −70°C. Once an aliquot is thawed, keep it at 4°C for no more than 1 wk.
16. Anti-BrdU monoclonal antibody (B44, Becton-Dickinson, cat. no. 347580).
17. TO-PRO-3 (Molecular Probes, Invitrogen cat. no. T3605).
18. Thymidine-deficient *Escherichia coli* MG1693 (*E. coli* stock center, http://cgsc.biology.yale.edu/top.html, CGSC#: 6411).
19. *lag-2*::GFP (green fluorescent protein) (*Caenorhabditis* Genetics Center (CGC), http://www.cbs.umn.edu/CGC/strains, strain #JK2868).
20. *lim-7*::GFP (*Caenorhabditis* Genetics Center (CGC), http://www.cbs.umn.edu/CGC/strains, strain #DG1575).

2.2.2 Web Resources

1. Reinke microarray data: http://cmgm.stanford.edu/~kimlab/germline/.
2. *in situ* RNA expression database: http://nematode.lab.nig.ac.jp/.
3. *C. elegans* site: http://elegans.swmed.edu/.
4. WormBase: http://www.wormbase.org/.
5. *C. elegans* strain collection (CGC): http://www.cbs.umn.edu/CGC/.
6. WormBook: http://www.wormbook.org/.
7. WormAtlas: http://www.wormatlas.org/index.htm.

2.3 Methods

2.3.1 Identification of Proliferating Cells in Wild-Type C. elegans Hermaphrodites

The *C. elegans* germline is composed of a U-shaped tube containing approx. 1000 germ cells in different states of differentiation. The mitotic germ cells, including GSCs, reside at one end, adjacent to the somatic DTC. The germline can be observed

in living animals (*see* **Subheading 2.3.1.1** and **Fig. 2.2A**). The organization of proliferative and meiotic cells is similar in hermaphrodites and males. The *C. elegans* hermaphrodites are self-fertile XX animals that first make sperm, then oocytes, whereas males are XO animals that make only sperm.

In wild-type young adult hermaphrodites (*see* **Note 1**) the mitotic region is approx. 20 cell diameters in length and contains approx. 225–250 germ cells. The length of the mitotic region is defined as the number of cell diameters between the DTC and the transition zone (TZ) ***(10,12)***. The mitotic region was initially defined by the positions of mitoses; however, now we know it is likely also to contain germ cells that have switched to early stages of meiosis (*see* **Subheading 2.3.3**, **Fig. 2.6**, and **refs. *10*** and ***12***). The TZ contains nuclei in early meiotic prophase; when stained with DAPI, the DNA in these nuclei has a distinctive crescent shape ***(16)*** (crescents; *see* **Fig. 2.2**, **Subheadings 2.3.1.2** and **2.3.1.4**). Most nuclei between the DTC and the TZ are proliferating; however, approx. 50 germ cells in the most

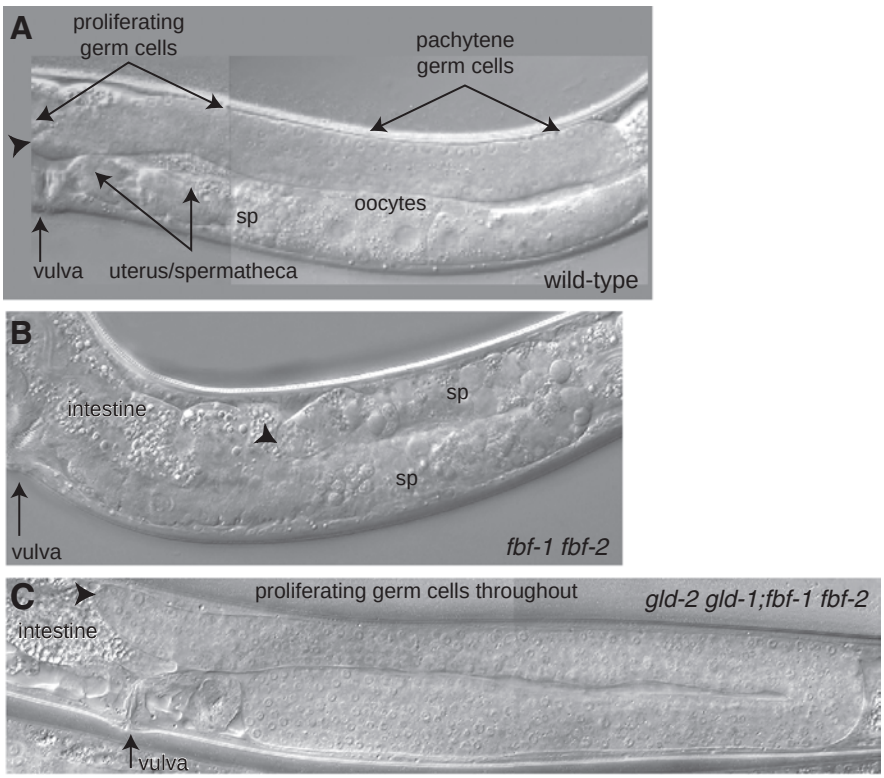

Fig. 2.2 Microscopy of wild-type and mutant germ lines. **A** Differential interference contrast (DIC) micrograph of a wild-type adult hermaphrodite. **B** DIC micrograph of an *fbf-1 fbf-2* double-mutant germline. Mature sperm are seen at the distal end. **C** DIC micrograph of a *gld-2 gld-1; fbf-1 fbf-2* mutant germline containing only mitotic germ cells