Mesenterial adult stem-like cells as a potential source of germinal and somatic lineages in a sea anemone

Paula Miramón-Puértolas

Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

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Faculty of Mathematics and Natural Sciences

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Para Maite y Jesús

Contents

Abstract

Adult stem cells (ASCs) sustain growth and homeostasis throughout the lifetime of animals by continuously supplying new cells to the organism. ASCs have been well characterized in vertebrate, nematode and insect models, which present tissue-specific stem cell populations with lineage-restricted potentials (e.g. intestinal, hematopoietic, neuronal, epithelial, germinal stem cells). In these organisms, the separation of germline stem cells from somatic lineages is a key step during embryonic development, which has long been thought to be common among metazoans. However, studies on a few other bilaterian and non-bilaterian model organisms have revealed the presence of ASCs bearing both germinal and somatic potentials throughout the lifetime of the organism (e.g. neoblasts in planarians, interstitial stem cells in hydrozoan cnidarians). The lack of data on ASCs from most animal phyla however hampers our understanding of the evolution of animal stem cells and germline segregation. Within cnidarians, ASCs have so far only been characterized in hydrozoan cnidarians, which present pluri- or multipotent interstitial stem cells (i.e. i-cells) that give rise to neurons, gland cells, cnidocytes and gametes. A longstanding question is whether i-cells are a hydrozoan-specific trait or ancestral to cnidarians. In my thesis, I aim to contribute to our understanding of cnidarian and animal stem cell evolution by identifying and characterizing ASCs in an anthozoan cnidarian, the sea anemone *Nematostella vectensis*. To find ASCs in *Nematostella*, I investigated the spatial expression of conserved germline and multipotency marker gene orthologs (e.g. *piwi*, *vasa*) in juvenile and adult polyps, and performed lineage tracing and single-cell RNA sequencing (scRNA-seq) to unveil their molecular profiles and potentials.

By combining and analysing Vasa2 immunostaining with *vasa2* and *piwi1* transgenic reporter lines in juveniles and adults, I characterized a population of mesenterial, extra-gonadal Vasa2+/Piwi1+ stem-like cells and their gonadal Vasa2+/Piwi1+ germinal progeny. Strikingly, I found that Vasa2+/Piwi1+ stem-like cells also give rise to abundant, gastrodermal somatic progeny cells, partially consisting of *soxB(2)*-expressing neuronal progenitor cells. Using scRNA-seq on tissue-enriched samples, I was able to identify cell clusters with partially overlapping expression profiles

corresponding to (1) the mesenterial Vasa2+/Piwi1+ stem-like cell population, (2) meiotic oogonia, (3) cells in M-phase, (4) *soxB(2)*+ neural progenitors and (5) cnidoglandular tract progenitor cells.

Altogether, my results suggest that the mesenterial Vasa2+/Piwi1+ stem-like cell population consists of multipotent ASCs with both germinal and somatic (incl. neuronal) potential. As such cells likely contribute to growth, tissue homeostasis and reproduction during juvenile and adult stages in *Nematostella* polyps, my thesis opens the door to a better characterisation of the cellular processes underlying whole-body regeneration, asexual reproduction, and body size plasticity in cnidarians. The shared potential, expression of genetic markers and cellular features with hydrozoan i-cells lead us to propose that Vasa2+/Piwi1+ ASCs with mixed germinal and somatic potential are an ancestral trait of cnidarians.

Abstrakt

Adulte stamceller (ASC) opprettholder vekst og homeostase gjennom hele livet til dyr ved kontinuerlig å tilføre nye celler til organismen. ASC-er har blitt godt karakterisert i vertebrat-, nematode- og insektmodeller, som presenterer vevsspesifikke stamcellepopulasjoner med avstamningsbegrenset potensial (f.eks. intestinale, hematopoetiske, nevronale, epiteliale, germinale stamceller). I disse organismene er separasjonen av kimstamceller fra somatiske linjer et nøkkeltrinn under embryonal utvikling, som lenge har vært antatt å være vanlig blant metazoer. Imidlertid har studier på noen få andre bilateriske og ikke-bilateriske modellorganismer avslørt tilstedeværelsen av ASC-er som bærer både germinalt og somatisk potensial gjennom hele organismens levetid (f.eks. neoblaster i planarier, interstitielle stamceller i hydrozoiske cnidarier) Mangelen på data om ASC-er fra de fleste dyrefyla hemmer imidlertid vår forståelse av utviklingen av dyrestamceller og kimlinjesegregering. Innenfor cnidarier har ASC så langt bare blitt karakterisert i hydroziske cnidarier, som presenterer pluri- eller multipotente interstitielle stamceller (dvs. i-celler) som gir opphav til nevroner, kjertelceller, cnidocytter og gameter. Et langvarig spørsmål er om i-celler er en hydrozoa-spesifikk egenskap eller fra forfedre til cnidaria. I oppgaven min har jeg som mål å bidra til vår forståelse av utviklingen av cnidaria og dyrestamceller ved å identifisere og karakterisere ASCs i en antozoisk cnidarie, sjøanemonen *Nematostella vectensis*. For å finne ASC-er i Nematostella, undersøkte jeg det romlige uttrykket av konserverte markørgener for kimlinje- og multipotens ortologer (f.eks. *piwi*, *vasa*) i juvenile og voksne polypper, og utførte avstamningssporing og enkeltcellet RNA-sekvensering (scRNA-seq) for å avdekke deres molekylære profiler og potensialer.

Ved å kombinere og analysere Vasa2 immunfarging med *vasa2* og *piwi1* transgene reporterlinjer i unge og voksne, karakteriserte jeg en populasjon av mesenteriale, ekstragonadale Vasa2+/Piwi1+ stam-lignende celler og deres gonadale Vasa2+/Piwi1+ germinal avkom. Påfallende nok fant jeg også at Vasa2+/Piwi1+ stam-lignende celler gir opphav til rikelig med gastrodermale celler av somatiske avstamning, delvis bestående av *soxB(2)*-uttrykke neuronale stamceller. Ved å bruke scRNA-seq på

vevsanrikede prøver, var jeg i stand til å identifisere celleklynger med delvis overlappende ekspresjonsprofiler tilsvarende (1) mesenterial Vasa2+/Piwi1+ stam-lignende cellepopulasjon, (2) meiotisk oogonia, (3) celler i M -fase, (4) *soxB(2)*+ nevrale forløpere og (5) cnido-glandulære traktat-forløpere.

Til sammen antyder resultatene mine at den mesenteriale Vasa2+/Piwi1+ stam-lignende celle-populasjonen består av multipotente ASC-er med både germinalt og somatisk (inkl. neuronalt) potensial. Siden slike celler sannsynligvis bidrar til vekst, vevs-homeostase og reproduksjon under unge og voksenstadier i Nematostella-polypper, åpner oppgaven min for en bedre karakterisering av de cellulære prosessene som ligger til grunn for helkropps-regenerering, aseksuell reproduksjon og plastisitet i kroppsstørrelse hos cnidarier. Det delte potensialet, uttrykket av genetiske markørene og cellulære funksjonene med hydrozoa i-celler, fører oss til å foreslå at Vasa2+/Piwi1+ ASC-er med blandet germinalt og somatisk potensiale er en egenskap fra forfedre for cnidarier.

List of publications

Paper I

Miramón-Puértolas, P., Steinmetz, P.R.H. (in preparation). An adult stem-like cell population contributes to germinal and somatic lineages in the sea anemone *Nematostella vectensis.*

Paper II

Miramón-Puértolas, P., Lebouvier, M., Saudemont, B., Loe-Mie, Y., Plessier, F., Marlow, H., Steinmetz, P.R.H. (in preparation). Mesentery-enriched single-cell atlas of *Nematostella vectensis* uncovers complex cell composition and a stem-like cell population.

Chapter 1: General introduction

1. Stem cells constitute the foundation of multicellular organisms

Stem cells are undifferentiated or, in rare cases, differentiated cells (e.g. *Hydra* epidermal stem cells) that have the unique ability to self-renew and give rise to progenitor cells that will divide and differentiate into new, functionally mature cells. The presence of stem cells is a universal trait among multicellular organisms (e.g. animals, plants, fungi).

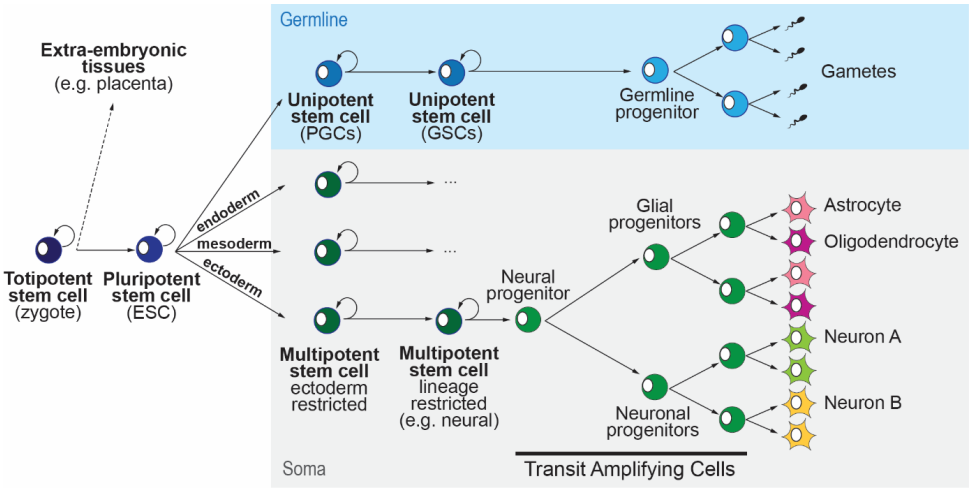


Figure 1. The different potencies of stem cells and their derivatives in humans. Schematic depicting the zygote, which holds the highest level of potency, and their derived embryonic stem cell lineages (e.g. germinal, endodermal, mesodermal and ectodermal). The germline exemplifies a unipotent stem cell lineage (e.g. male germline). The ectodermally derived neural stem cells exemplify multipotency, as they give rise to a variety of cell types. Multipotent stem cells give rise to progenitor cells, also named transit-amplifying cells (e.g. neural progenitors), which undergo one to several rounds of division before giving rise to terminally differentiated cells (e.g. astrocytes, oligodendrocytes, neurons). ESC: embryonic stem cell, PGC: primordial stem cell, GSC: germline stem cell.

In animals, stem cells have been described as small and round with a high nuclear to cytoplasmic ratio, a prominent nucleolus and a high ribosomal content (Gupta and Santoro, 2020; Han et al., 2020; Zakrzewski et al., 2019). Stem cells are classified according to their potential, which depends on the diversity of differentiated cell types that they can give rise to **(Figure 1)**. The highest level of potency, totipotency, is held

by the zygote, which can differentiate into embryonic and extra-embryonic tissues (if existing). Embryonic stem cells (ESCs) are considered pluripotent, as they can give rise to all the germ cell layers of the embryo (i.e. all cell types of the organism) but not to extra-embryonic tissues. When extra-embryonic tissues are missing, pluripotency and totipotency are thus used synonymously. Multipotent stem cells can give rise to a limited range of cell types, often connected by lineage (e.g. hematopoietic stem cells, neural stem cells). Finally, unipotent stem cells can give rise to a single cell type (e.g. epithelial stem cells, germline stem cells).

Stem cells play a crucial role during embryonic development as new organs and tissues form. Certain populations of stem cells are retained after embryogenesis or larval development (if present) and are commonly referred to as "adult stem cells" (ASCs) (Zakrzewski et al., 2019). While named “adult”, these stem cells are defined as being found not only at adult but also at juvenile life stages and constitute a main source for growth, regeneration and tissue homeostasis (Clevers and Watt, 2018; Wagers and Weissman, 2004). In vertebrates, insects and nematodes, ASCs are often found in relatively small numbers in a well-defined niche, which is a microenvironment that tightly regulates and maintains their stemness (Drummond-Barbosa, 2008; Ferraro et al., 2010). Other animals, such as planarians, acoels, sponges and cnidarians, present more abundant ASCs that seem to lack a defined niche (Bosch et al., 2010; Martinez et al., 2022; Rossi and Salvetti, 2019).

A stem cell divides asymmetrically, generating a daughter cell that retains the ability to self-renew and a more committed progenitor cell that can be multipotent or unipotent **(Figure 1)**. Progenitor cells cannot self-renew, performing a finite number of divisions usually accompanied by a progressive reduction of their potential. While proliferating, progenitor cells migrate into a tissue where they will eventually differentiate into defined cell types. By dividing, progenitor cells amplify cell numbers and are thus also called transit-amplifying cells (TAC) **(Figure 1)** (Hsu et al., 2014).

2. The germinal potential is set aside for sexual reproduction

Multicellular organisms specify a set of cells known as germ cells or gametes (eggs in

females and sperm in males) that will carry the heritable information to the next generation. The germline, the cell lineage giving rise to gametes, derives from unipotent germline stem cells (GSCs) (Lehmann, 2012) **(Figure 1)**. During sexual reproduction, gametes fuse at fertilization to generate a totipotent zygote that develops into a new organism. The potential of GSCs to generate a whole new organism is preserved by repressing somatic differentiation (Strome and Updike, 2015). This idea was already born in 1892, when August Weismann hypothesized that germline cells, as carriers of the hereditable information, must contain a unique set of determinants (i.e. the "germ plasm") absent in somatic lineages (Weismann, 1892). According to Weismann these determinants are carried by the germline and transferred to the zygote, and again to the germline of the next generation in a continuous manner. In his germ plasm theory, Weismann defined thus a physical barrier between the soma and the germline, in which the germline can give rise to all somatic cell types through fertilization, but somatic lineages cannot give rise to the germline. According to his theory, the genetic information carried by the germline cannot be altered by somatically acquired mutations.

2.1. Traditional postulations on embryonic germline segregation

Weismann's hypothesis was confirmed by the discovery of molecular determinants directly transferred from the egg to the germline of the embryo in chrysomelid beetles (Hegner, 1908; Hegner, 1914). This mode of germline segregation, named preformation **(Figure 2A)**, has since been found and described in detail in many genetic model organisms, for example *D. melanogaster*, *C. elegans* and *X. laevis* (Extavour and Akam, 2003; Sagata et al., 1981; Strome and Updike, 2015; Williamson and Lehmann, 1996; Wolf et al., 1983). In these animals, the egg carries maternal determinants that are asymmetrically distributed during early cleavages and transferred to a set of early ESCs, called primordial germ cells (PGCs), which will acquire germline identity. During embryonic development, these PGCs migrate to the emerging gonad and establish a population of GSCs (Richardson and Lehmann, 2010). ESCs not receiving maternal determinants acquire restricted, somatic fate and give rise to the diverse somatic lineages composing the organism. Mammalian embryos display

a different mode of segregation called epigenesis, during which a subset of ESCs, considered somatic stem cells, are induced by neighbouring cells to become PGCs at perigastrulation stage **(Figure 2B)** (Ewen-Campen et al., 2010; Seervai and Wessel, 2013; Tang et al., 2016). Therefore, the epigenetic segregation of the germline breaks the continuity of the germline and its determinants, contradicting the germ plasm theory. For this reason, Weismann's theory has been for many years a subject of controversy (Extavour and Akam, 2003; MacCord and Duygu Ozpolat, 2019; Raz and Yamashita, 2021; Solana, 2013).

During the XXth century, most genetic model organisms (e.g. insects, nematodes, vertebrates) were shown to segregate the germline during embryogenesis, whether it was by preformation or epigenesis. Thus, embryonic germline segregation was assumed to be a key step during animal embryonic development. It was only when organisms such as planarians, acoels, hydrozoan cnidarians and sponges were put in the spotlight that instances of post-embryonic germline segregation were found, leading to a revision of the germline concept.

2.2. Post-embryonic segregation and reimagining the germline

In recent years, diverse bilaterian and non-bilaterian animals have been found to show post-embryonic segregation of the germline (e.g. acoels, planarians, cnidarians, sponges). In these animals, ASCs traditionally considered of somatic nature, give rise not only to somatic lineages but also to the germline **(Figure 2C)**. Increasing evidence for the presence of germ plasm determinants in ASCs has led to question their presumed somatic identity and to reconsider the germline concept, resulting in the primordial stem cell hypothesis (Solana, 2013). This hypothesis presents a concept of the germline that includes stem cells with not only germinal but also somatic potential, namely primordial stem cells (PriSCs). According to this hypothesis, PriSCs are conserved across the animal tree, share a set of genetic determinants (the "germ plasm") and are set aside during embryogenesis in all animals. In the case of embryonic germline segregation by preformation, PriSCs are considered as transient and quickly restrict to become PGCs early during embryogenesis (**Figure 2D**; e.g. *D. melanogaster, C. elegans*). In the case of embryonic segregation by epigenesis, mammalian ESCs

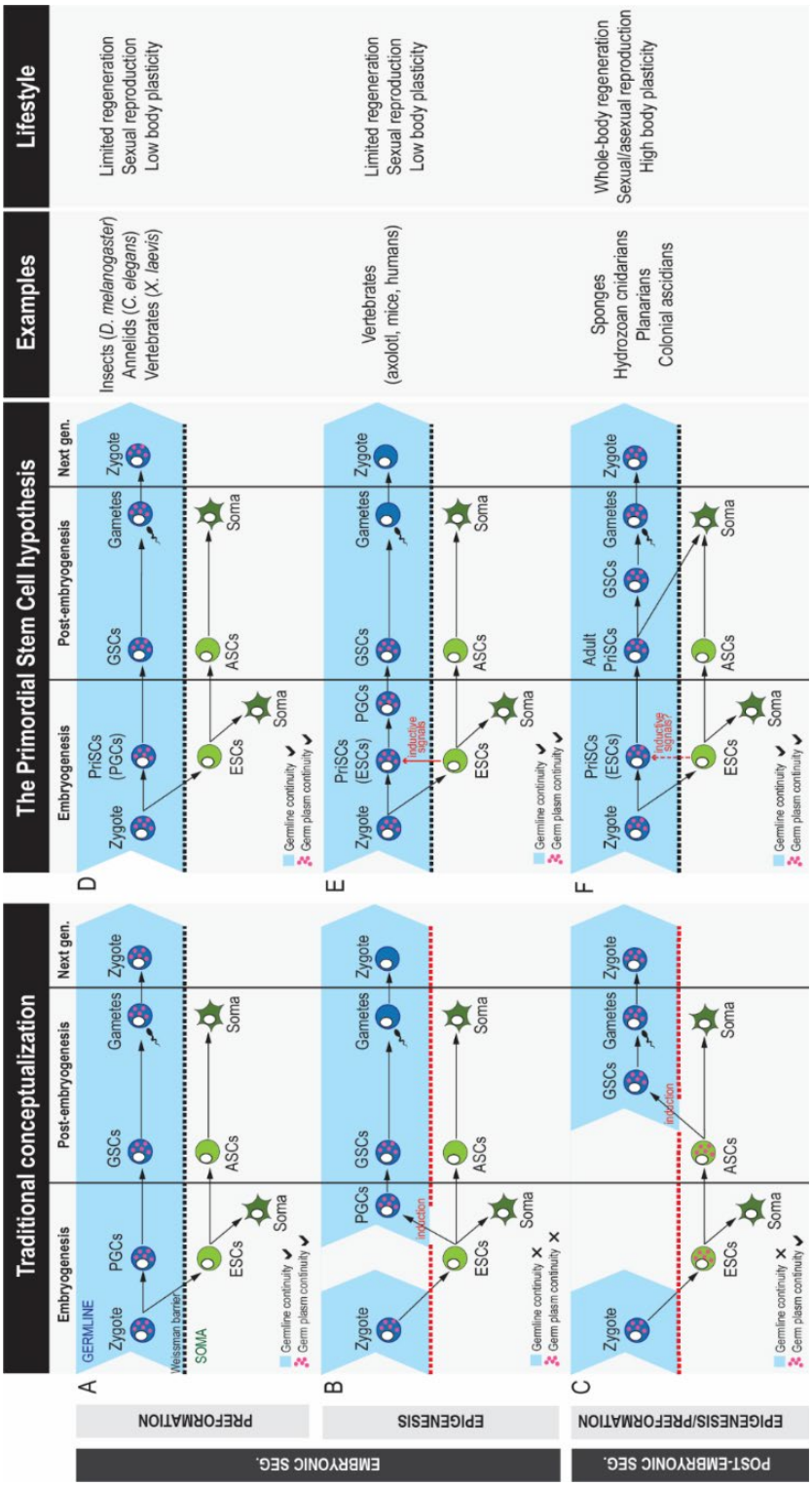


Figure 2. Redefining the germline concept: the primordial stem cell hypothesis. Table illustrating the modes of embryonic and post-embryonic germline segregation from the traditional point of view (A-C) and according to the primordial stem cell hypothesis (D-F) (Solana 2013). Cells giving rise to the germline during epigenetic embryonic segregation and post-embryonic segregation are not somatic stem cells as traditionally assumed, but primordial stem cells (PriSCs) holding germinal and somatic potential. Schematics based on Solana 2013.

traditionally thought to be of somatic nature, such as the inner cell mass (ICM), present certain germline genetic determinants before being induced to become PGCs. Thus, the ICM can be considered as PriSCs, a subset of which become restricted to the germline by inductive signals during embryogenesis **(Figure 2E)**. In the case of post-embryonic germline segregation, PriSCs are also set aside by preformation or epigenesis during embryogenesis but retain dual germ/soma potential throughout the lifetime of the organism **(Figure 2F)**. Examples of adult PriSCs (i.e. ASCs with germ/soma potential) are planarian and acoel neoblasts, hydrozoan i-cells and sponge choanocytes and archaeocytes (Bode, 1996; Funayama, 2013; Srivastava et al., 2014; Wagner et al., 2011). The conceptualization of PriSCs keeps the continuity of the germline intact through generations, reconciliating the germ plasm theory of Weismann with the different modes of germline segregation described in animals.

2.3. Germ cells develop and mature during gametogenesis

After germline segregation takes place, PGCs must find their way into the gonad, the tissue providing the right environment for GSCs to settle and for gametogenesis to take place. Gametogenesis is the process during which GSCs give rise to haploid, mature eggs during oogenesis or sperm cells during spermatogenesis. GSCs asymmetrically divide and give rise to new GSCs and progenitor cells (i.e. oogonia or spermatogonia), which will mitotically divide a limited number of times and eventually start to differentiate into gametes **(Figure 3)**.

2.3.1. Meiosis produces haploid germ cells

The first step during gamete differentiation is meiosis, a process of cell division that produces haploid germ cells. Meiosis encompasses two cycles of cell division: during meiosis I homologous chromosomes segregate and in meiosis II sister chromatids are separated **(Figure 3)** (Page and Hawley, 2003). An important process of meiosis I is the recombination between homologous chromosomes, which generates new allele combinations. Recombination takes place during prophase I thanks to the assembly of the synaptonemal complex and other molecular mechanisms highly conserved among eukaryotes (Jessus et al., 2020; Lenormand et al., 2016; Ramesh et al., 2005).

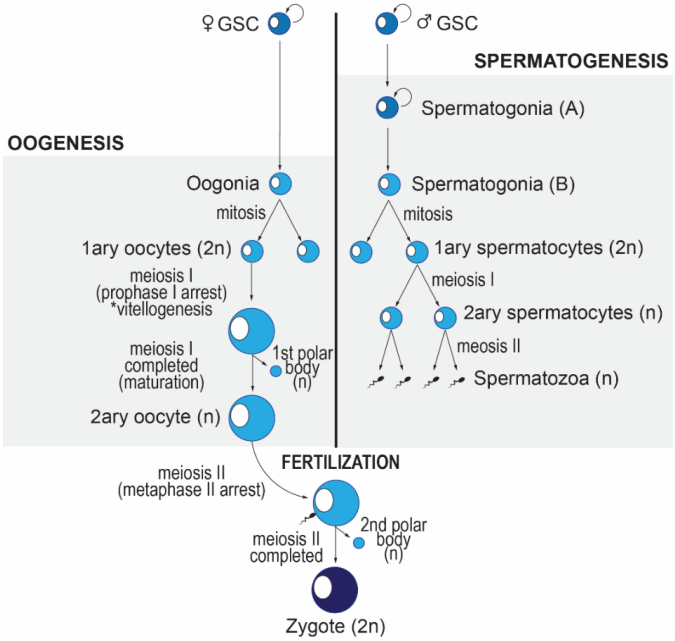


Figure 3. The cellular lineages of female and male gametogenesis. Schematic depicting oogenesis and spermatogenesis, exemplified as in an oviparous vertebrate species (e.g. *Xenopus*) whose oocytes undergo vitellogenesis while arrested in prophase I and stay in metaphase II arrest until fertilization. 2n: diploid; n: haploid.

2.3.2. Oogenesis

When an oogonium starts meiosis, it is called a primary oocyte. One of the key steps in oogenesis across animals is the prophase I arrest at diplotene stage, when the synaptonemal complex disassembles and homologous chromosomes start to separate, remaining linked only by chiasmata (Jessus et al., 2020; Page and Hawley, 2003). In humans, oocytes remain arrested until reaching puberty, when one oocyte resumes meiosis in each menstrual cycle in a process called oocyte maturation **(Figure 3)**. In oviparous species, oocytes undergo vitellogenesis during meiotic arrest, accumulating reserves for the zygote in their cytoplasm until a certain size is reached and meiosis resumes. Oocytes produce and discard one polar body containing half of the genetic content and very little cytoplasm after each meiotic cell division **(Figure 3)**. Mature eggs are released during ovulation. In vertebrates, mature eggs are often arrested at metaphase II and only complete meiosis II upon fertilization, releasing a second polar

body **(Figure 3)**. While meiotic arrest is a highly conserved aspect of oogenesis, the mechanisms that regulate arrest maintenance, oocyte growth and maturation vary considerably between species (Jessus et al., 2020). Oogenesis takes place in the female gonad (i.e. ovary), which is often composed of somatic cells that support oocyte growth (i.e. nurse cells) and gland cells that regulate oocyte maturation and ovulation by secreting hormones (Haccard and Jessus, 2006; Jessus et al., 2020).

2.3.3. Spermatogenesis

During vertebrate spermatogenesis, adult GSCs are known as spermatogonia type A, which self-renew and give rise to spermatogonia type B, which mitotically divide until generating primary spermatocytes that undergo meiosis **(Figure 3)**. In adult males, meiosis is not arrested and produces four mature sperm cells at every meiotic event. In some species, such as humans, the cell cycle of spermatogonia type A and B is paused until reaching puberty, when cell division resumes. Spermatogenesis takes place in the testes or spermaries, the male gonads, which can vary in complexity among species but usually present a sack-like or tubular shape, with spermatogonia localizing in the periphery and the maturation process taking place towards the centre, where mature spermatids are found (Fritz, 1986; Leonard and Cordoba-Aguilar, 2011).

3. The molecular signature of germline stem cells and primordial stem cells

Gene expression and functional studies have revealed a set of highly conserved genetic markers which are consistently enriched and required in PGCs, GSCs and PriSCs across the animal tree (Alié et al., 2015; Ewen-Campen et al., 2010; Fierro-Constaín et al., 2017; Juliano et al., 2010; Rinkevich et al., 2022). This gene set, comprising *vasa*, *pl10*, *piwi*, *tudor*, *nanos*, *pumilio* and *bruno* genes, has been proposed as "germline multipotency program" (GMP) to maintain stemness in these cells (Fierro-Constaín et al., 2017; Juliano et al., 2011). A subset of GMP genes consisting of *vasa*, *pl10*, *piwi* and *tudor* encode for proteins that aggregate in granules around the nucleus of PGCs, GSCs and PriSCs (Gao and Arkov, 2013). Long before their molecular composition was elucidated, the presence of such granules was discovered in the germline of insects

(Hegner, 1908; Mahowald, 1962), being the first experimental evidence supporting the existence of a “germ plasm” as hypothesized by Weismann (Weismann, 1892).

3.1. Germ granules: discovery, composition, and function

Germ granules were discovered in 1914 as strongly stained granular inclusions in the cytoplasm of chrysomelid beetle eggs, locating to the posterior pole and being directly transferred into early germ cells (Hegner, 1908; Hegner, 1911; Hegner, 1914). Removing such granules led to the absence of germ cells in the embryos and thus it was assumed that they contained germ cell determinants (Hegner, 1908). Electron microscopy revealed that the granular inclusions consisted of aggregates of electron-dense fibrils (also termed *nuage*) associated with the cytoplasmic side of the nuclear membrane (André and Rouiller, 1956; Eddy, 1974). These aggregates were discovered simultaneously in different species and contexts, which lead to the coining of different terms: polar granules at the posterior egg pole of several *Drosophila* species (Mahowald, 1962; Mahowald, 1968; Mahowald, 1971a; Mahowald, 1971b), cortical granules in amphibian eggs (Balinsky, 1966; Eddy and Ito, 1971; Kotani et al., 1973; Williams and Smith, 1971), *nuage* in spider eggs (André and Rouiller, 1956) and rat embryos (Eddy, 1974), P-granules in the *C. elegans* embryonic P-lineage (Wolf et al., 1983), and the chromatoid body in mammalian post-meiotic spermatids (Brunn, 1876; Burgos and Fawcett, 1955; Parvinen, 2005; Söderström, 1981). For clarity, I will employ the term 'germ granules' for these aggregates throughout this work.

The size of the granules varies between organisms (up to 1µm), while their electron-dense fibrils have a rather conserved diameter of 10-15nm. The composition of germ granules has been thoroughly dissected in several model organisms (reviewed in Gao and Arkov, 2013). They consist of one of several types of ribonucleoprotein (RNP) complexes (e.g. stress granules) that can be found in the cytoplasm. The RNA components are mRNAs, non-coding RNAs such as Piwi-interacting RNAs (piRNAs), and mitochondrial ribosomal RNAs. Among the proteins, some components conserved across animals include Piwi family proteins, Tudor domain-containing proteins, and RNA helicases such as Vasa and PL10 proteins (Gao and Arkov, 2013).

Germ granules are not only found on the nuclear envelope but often appear in association with highly dynamic, transient organelle aggregates called the "mitochondrial cloud" or Balbiani body (Bb), composed of thousands of mitochondria and sometimes comprising Golgi, endoplasmic reticulum (ER) cisternae and centrioles (Kloc et al., 2014; Meikar et al., 2014). The Bb has been described during gametogenesis in oocytes and post-mitotic spermatids of many animals, including *Xenopus*, zebrafish, mammals and a diversity of arthropods (Kloc et al., 2004; Kotaja and Sassone-Corsi, 2007; Meikar et al., 2011; Pepling et al., 2007). These aggregates show a great divergence in their morphology, cell location and dynamics even within closely related species. Their detailed functions and assembly mechanisms remain poorly understood.

While traditionally linked to PGCs and GSCs, there is increasing evidence for the presence of perinuclear germ granules also in adult PriSCs such as hydrozoan i-cells (Noda and Kanai, 1977) and planarian neoblasts (Hori, 1982). The study of the molecular composition of germ granules in different animals has confirmed that they are indeed a conserved feature of stem cells holding germinal potential (i.e. PriSCs, PGCs and GSCs) across Metazoa (Juliano et al., 2010; Solana, 2013; van Wolfswinkel, 2014).

3.1.1. The RNA helicases Vasa and PL10

RNP complexes are dynamic and in constant remodelling due to the presence of RNA helicases, which unwind double-stranded RNAs in an ATP-dependent way. Vasa and PL10 belong to the DEAD-box (Asp-Glu-Ala-Asp) protein family, characterized by nine conserved sequence motifs important for the ATP-dependant catalytic activity of the helicase (Linder and Jankowsky, 2011; Linder and Lasko, 2006). The DEAD box family of RNA helicases presents a very ancient lineage, with members found in all eukaryotic cells, many bacteria and even archaea, where they are required for RNA metabolism and gene expression control (Linder and Jankowsky, 2011).

The RNA helicase Vasa is only found in metazoans, where it is a highly conserved germ granule component and plays a crucial role in GSC maintenance and

gametogenesis (Extavour, 2005; Fabioux et al., 2004; Hartung et al., 2014; Hay et al., 1988; Qiu et al., 2013; Raz, 2000; Rosner et al., 2009; Roussell and Bennett, 1992; Shimaoka et al., 2017; Spike et al., 2008). Its relevance for germ cell determination varies depending on the species and sex. In *D. melanogaster,* Vasa is necessary for female germ cell specification and oocyte development but not for spermatogenesis (Styhler et al., 1998). In mice, Vasa is required for male, but not female fertility (Tanaka et al., 2000). Vasa is also enriched in adult PriSCs such as planarian neoblasts and hydrozoan i-cells (Mochizuki et al., 2001; Rebscher et al., 2008; Wagner et al., 2012). It plays important roles during embryonic and post-embryonic development, acting for instance as a mitotic regulator during early cleavages in sea urchins and spiders (Gustafson and Wessel, 2010; Schwager et al., 2015; Yajima and Wessel, 2011; Yajima and Wessel, 2015).

There is little data on the molecular mechanisms underlying the functions of Vasa across species. Studies in *D. melanogaster* have shown that Vasa can bind the eukaryotic initiation factor 5B (eIF5B), which recruits the 60S ribosomal subunit for translational initiation of maternal transcripts. In addition, it specifically binds and promotes the translation of mRNAs such as *mei-P26*, whose protein product represses certain miRNAs in order to promote germ cell identity (Johnstone and Lasko, 2004; Liu et al., 2009). These observations suggest that Vasa acts as a translational regulator of certain mRNAs by shaping their secondary structures and facilitating their interaction with accessory proteins (Styhler et al., 1998). Vasa is subject to strong post-translational regulation leading to big variances of cellular mRNA and protein levels (Yajima and Wessel, 2015).

PL10 helicases are considered to have an earlier evolutionary origin than Vasa, as PL10 orthologs can be found in a wide range of eukaryotes, including fungi, plants and animals (Chang and Liu, 2010; Linder and Jankowsky, 2011). Some species possess several PL10 homologues, which can show ubiquitous or exclusively germinal expression. PL10 orthologs have been described to play important roles in cell cycle regulation, differentiation, survival and apoptosis (Sharma and Jankowsky, 2014). They are not only required in the germline but also in adult PriSCs (Mochizuki et al.,

2001; Shibata et al., 1999; Solana and Romero, 2009) and in somatic lineages such as the neural crest (Perfetto et al., 2021). It has been hypothesised that Vasa helicases derive from a PL10-related gene that restricted its expression and function to the germline.

3.1.2. Piwi proteins and Piwi/Tudor complexes

Piwi proteins are part of the PIWI subfamily, which together with the AGO subfamily belong to the evolutionary conserved Argonaute/PIWI protein family. While the AGO subfamily is found in fungi and plants, PIWI subfamilies are likely restricted to animals (Mochizuki et al., 2002). Thus, while the origin of the AGO protein family likely predates the evolution of multicellularity, the PIWI subfamily is considered a metazoan innovation (Alié et al., 2015; Kerner et al., 2011).

Piwi and Ago proteins share a conserved PAZ domain near the N-terminal, which binds small noncoding RNAs (ncRNAs), and a C-terminal PIWI domain, which presents cleaving activity. ncRNAs guide Ago/Piwi-containing protein complexes to RNA target sites to regulate gene expression. While Ago proteins bind microRNAs (miRNAs) and small interfering RNAs (siRNAs), Piwi proteins bind PIWI-interacting RNAs (piRNAs). piRNAs measure 24-32 nucleotides and derive from long single-stranded RNA precursors encoded by intergenic regions containing repetitive and transposable elements (Juliano et al., 2011; Thomson and Lin, 2009). Across metazoans, piRNAs are consistently enriched for transposon sequences (van Wolfswinkel, 2014). By binding Piwi proteins, piRNAs guide Piwi-containing complexes to target transposon mRNAs, which are cleaved by the Piwi endonuclease activity. In addition, complementary Piwi processing of the cleaved products leads to a ping-pong cycle that amplifies the piRNAs **(Figure 4)** (Czech and Hannon, 2016). Thus, Piwi proteins play a key role in protecting the nuclear genome through transposon silencing. Indeed, Piwi depletion leads to an increase in transposon mRNA expression (Li et al., 2021; Tóth et al., 2016).

The expression and intracellular location of *piwi* mRNAs are evolutionarily conserved among animals. *piwi* mRNA is consistently found in the germ granules of PriSCs,

GSCs, and developing gametes of *D. melanogaster*, zebrafish, *C. elegans*, mice, colonial ascidians, acoels, planarians, hydrozoan cnidarians, sponges and ctenophores (Juliano et al., 2011; van Wolfswinkel, 2014). Functional analyses have revealed the importance of Piwi proteins for GSC maintenance in males and females of *D. melanogaster* and *C. elegans* (Cox et al., 1998; Gonzalez et al., 2021; Megosh et al., 2006), while in mice Piwi is required for proper spermatogenesis (Kuramochi-Miyagawa et al., 2004). Piwi plays a key role in the maintenance of planarian neoblasts and colonial ascidian hemoblasts, and it is important for their role in regeneration (Kawamura and Sunanaga, 2011; Palakodeti et al., 2008; Reddien et al., 2005; Sunanaga et al., 2010). Altogether, Piwi proteins play a conserved key role in stem cell maintenance, germline determination and gametogenesis, which are processes that require transposon silencing to ensure genome integrity (Tóth et al., 2016; van Wolfswinkel, 2014). In addition to their function in transposon silencing, piRNAs also map to mRNAs (e.g. *nanos*) to facilitate Piwi-mediated post-transcriptional regulation (Collins and Penny, 2009; Rouget et al., 2010).

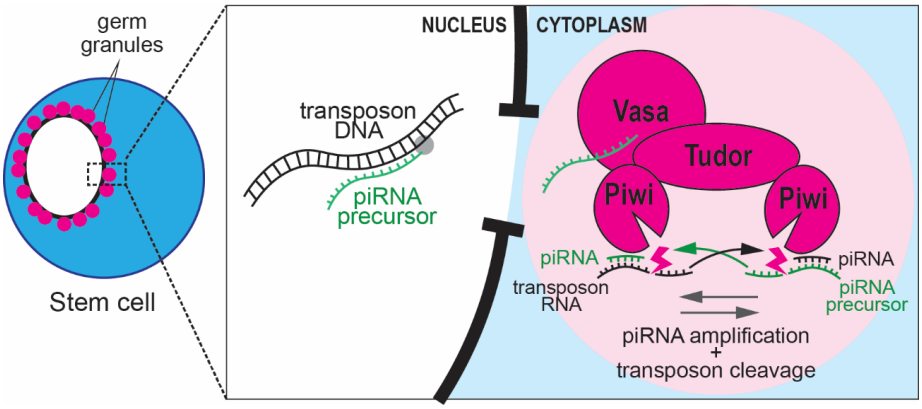


Figure 4. The role of germ granules in transposon silencing. Schematic of a close-up of the nuclear membrane of a stem cell, depicting a nuclear pore and an associated germ granule. The germ granule contains a protein complex composed of Vasa, Tudor and Piwi, which captures piRNAs coming from the nucleus. Piwi binds to piRNAs, which guide the complex to target RNA sequences (e.g. transposons) that are subsequently cleaved by Piwi. The cleaved product is used by Piwi as a guide to cleave piRNA precursors and generate new piRNAs, and so a 'ping-pong cycle' of piRNA amplification starts, resulting in effective transposon silencing. Adapted from Lin, 2012; van Wolfswinkel, 2014.

Piwi-piRNA complexes also play a role in somatic stem cells, where they are expressed at lower levels than in germline precursor cells (Ross et al., 2014). *piwi* orthologs are expressed for example in epithelial stem cells in *Hydra* (Juliano et al., 2014; Teefy et al., 2019) and in human hematopoietic stem cells (Sharma et al., 2001). Thus, Piwi proteins are expressed not only in PriSCs and GSCs but also in somatic stem cells, drawing a link between Piwi and cell potency (Li et al., 2021; Ross et al., 2014; van Wolfswinkel, 2014). Interestingly, *piwi* orthologs have also been shown to be expressed and required in certain differentiated cell types, such as neurons in the central nervous system of mice (Kim, 2019; Lee et al., 2011; Zhao et al., 2015).

Piwi proteins form complexes with Tudor domain-containing proteins, which bind to demethylated arginine residues at the N-terminus of Piwi and Vasa proteins. Multiple Tudor domains are usually found in the same protein. They are thought to act as scaffolding for germ granule assembly while regulating the identity of the piRNAs that Piwi proteins bind **(Figure 4)** (Huang et al., 2011; Mathioudakis et al., 2012; Vasileva et al., 2009). Piwi-Tudor complexes associate closely to the exterior of nuclear pores **(Figure 4)**. There, with the help of the nuclear helicase UAP56, Vasa transfers newly synthetized piRNAs from the nucleus to Piwi proteins, facilitating clamping and amplification of cytoplasmic piRNAs in the ping-pong cycle (Lin, 2012; Xiol et al., 2014; Zhang et al., 2012). Tudor domain-containing proteins have been consistently found in animal germ granules and, similarly to Vasa and Piwi, are required for GSC maintenance in *D. melanogaster* and for spermatogenesis in mice (Kirino et al., 2010; Kuramochi-Miyagawa et al., 2004; Megosh et al., 2006).

3.2. Other germline and multipotency markers: *nanos*, *pumilio* and *bruno*

The *nanos* gene encodes for another well characterized GMP component. Nanos functions as a transcriptional and translational repressor characterized by zinc-finger motifs. Its orthologs are found in all studied animal species (De Keuckelaere et al., 2018). Nanos has been strongly associated to PGC and GSC maintenance, but is also required to prevent stem cell differentiation during embryonic, larval and somatic development (Extavour and Akam, 2003; Mochizuki et al., 2000; Rinkevich et al., 2022; Wang and Lin, 2004). It forms complexes with the RNA-binding protein

Pumilio, another GMP component, to repress target mRNAs involved in gametogenesis and somatic differentiation and is thus important to maintain stemness (Kadyrova et al., 2007; Nakahata et al., 2001; Parisi and Lin, 1999; Salvetti et al., 2005; Sonoda and Wharton, 1999; Weidmann et al., 2016; Wharton et al., 1998). The requirement of *pumilio* in somatic stem cell maintenance has been characterized for neural and hematopoietic stem cells in mammals (Spassov and Jurecic, 2003; Yang et al., 2004; Zahr et al., 2018; Zhang et al., 2017).

The Bruno-like family consists of proteins containing RNA-recognition motifs (RRM), found in plants and animals, which repress target mRNAs by binding specific Bruno response elements (BRE) sequences (Chekulaeva et al., 2006; Good et al., 2000). They are expressed in *Drosophila* germ cells, planarian neoblasts, diverse embryonic and post-embryonic tissues of vertebrates and in ctenophore stem cells (Alié et al., 2011; Barreau et al., 2006; Good et al., 2000; Guo et al., 2006). Functions associated to Bruno proteins are also varied, and include germ cell development, stem cell maintenance, pre-mRNA processing and synaptic vesicle release (Barreau et al., 2006; Chekulaeva et al., 2006; Guo et al., 2006; Suzuki et al., 2002). In the zebrafish embryo, *bruno* mRNA has been described to localize in the *nuage* during early cleavage stages (Hashimoto et al., 2006).

4. The potency of adult stem cells is linked to animal life history traits

Embryonic and adult PriSCs, PGCs, GSCs and other stem cells across metazoans share highly conserved molecular and cellular signatures. Yet, the biology of animal stem cells exhibits key differences between species as shown by their diverse modes of germline segregation, reproduction, and regenerative potential. Stem cells have evolved in each lineage to adapt and achieve top fitness according to the species' lifestyle and environment.

4.1. Embryonic segregation of the germline and an expiring soma

Extensive studies have characterized germline segregation and ASCs in a diversity of vertebrate model organisms, all of which perform embryonic germline segregation either by preformation (e.g. zebrafish, chick and *Xenopus*) or epigenesis (e.g. axolotl

and mammals) (Extavour and Akam, 2003; Wen and Tang, 2019). Once PGCs are segregated, somatic ESCs gradually reduce their potential as they divide and become restricted to a specific germ layer and cell lineage, while organizing into organ primordia (Kallos, 2011). After embryogenesis, stem cell niches sustain populations of somatic ASCs (e.g. hematopoietic, intestinal, mesenchymal, epithelial) throughout the lifetime of the organism (Ferraro et al., 2010).

Among ecdysozoans (e.g. arthropods, nematodes), clear examples of embryonic germline segregation by preformation are found in well-researched model organisms such as *D. melanogaster* and *C. elegans*, as well as in crustaceans (e.g. amphipods and decapods) (Extavour, 2005; Qiu et al., 2013). Among arthropods, germline segregation by epigenesis has been described in honeybees, beetles (e.g. *Tribolium*), moths and butterflies, myriapods and chelicerates (Quan and Lynch, 2016). So far, all investigated ecdysozoans models display embryonic germline segregation, with separate GSCs and somatic, lineage-restricted ASCs (e.g. neuronal, intestinal) present after embryogenesis, as also found in vertebrate models.

The limited potential of ASCs in ecdysozoans and vertebrates is likely the basis of their limited body plasticity and the limited or absent potential to regenerate body parts in these animals (e.g. limb regeneration in arthropods and axolotl) **(Figure 5)** (Anderson et al., 2001; Rinkevich et al., 2022; Tanaka and Reddien, 2011). Asexual modes of reproduction involving regeneration such as budding or fragmentation are therefore absent in ecydozoans or vertebrates. Parthenogenesis has been described in some species of insects, reptiles, amphibians and fish (Fujita et al., 2020; Subramoniam, 2018). While in vertebrates and ecdysozoans, ASCs populations sustain organismal growth and homeostasis throughout their lifetime, the limited self-renewing capacity eventually leads to organismal decay (i.e. aging) and death (Drummond-Barbosa, 2008; Pathak and Banerjee, 2021).

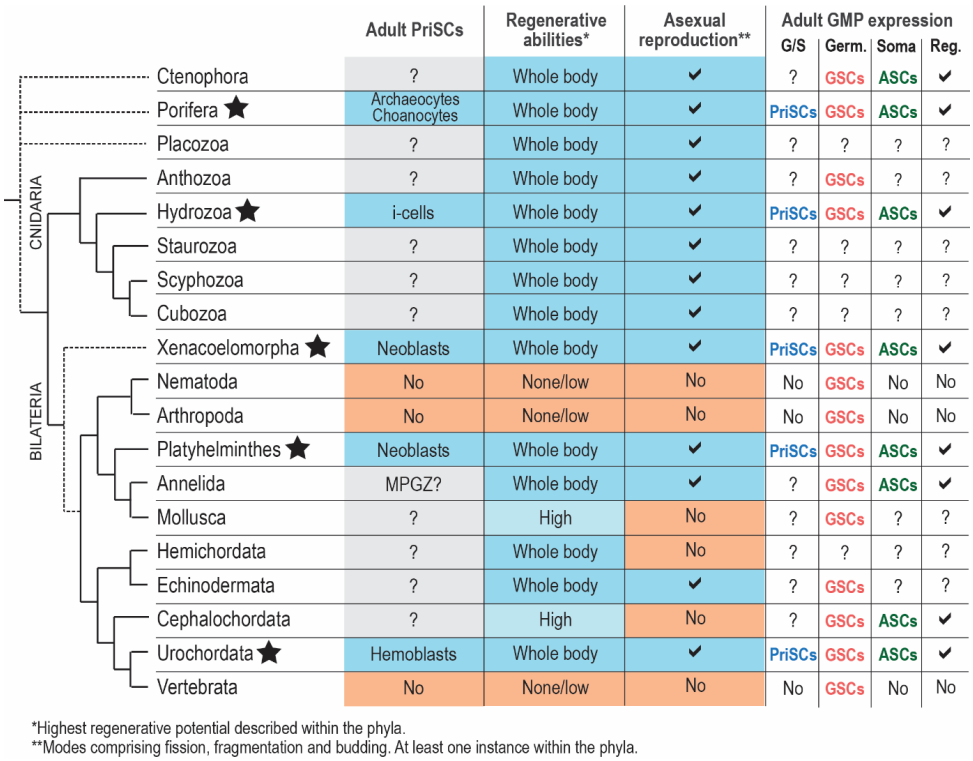
4.2. Adult PriSCs as a hallmark for body plasticity across Metazoa

4.2.1. Highly regenerative bilaterian organisms present adult PriSCs

In lophotrochozoans, stem cell research has focused on flatworms, annelids, and some

molluscs. Planarian flatworms (e.g. *S. mediterranea* and *D. japonica*) present an adult population of pluripotent stem cells called neoblasts that give rise to all somatic lineages and the germline (Baguna et al., 1989; Roberts-Galbraith and Newmark, 2015). Neoblasts underlie the ability of adult planarians to perform asexual reproduction by fission and their outstanding regenerative capacities (Gehrke and Srivastava, 2016; Wagner et al., 2011; Zeng et al., 2018). Recent efforts have focused on characterizing the heterogeneity of the neoblasts and the molecular pathways regulating their differentiation into different lineages (Zeng et al., 2018). Several annelid species (e.g. *P. dumerilii, C. teleta, A. virens, E. japonensis)* present a set of ASCs locating to the mesodermal posterior growth zone (MPGZ) that are molecularly similar to PGCs. These ASCs continuously generate new body segments and underlie the ability to perform posterior regeneration in annelids (Dill and Seaver, 2008; Gazave et al., 2013; Giani et al., 2011; Kostyuchenko, 2022; Rebscher et al., 2012; Sugio et al., 2008). In addition, some species like *P. leidyi* are able to reproduce asexually by fission (Del Olmo et al., 2022; Özpolat and Bely, 2015). Nevertheless, it remains unresolved if annelid ASCs hold germinal potential (Rebscher et al., 2012). Little data is available on ASCs in molluscs, but adult PriSCs giving rise to somatic and germinal lineages have been proposed to reside in the gut of bivalves (Milani et al., 2017).

Within cephalochordates, there is evidence for embryonic germline segregation in several species of *Amphioxus,* yet ASCs molecularly similar to PGCs have been associated to tail regeneration (Dailey et al., 2016). Among urochordates, the solitary ascidian *Ciona robusta*, segregates the germline during embryogenesis but can be replaced if ablated after metamorphosis. This observation suggests the presence of adult PriSCs that are able to segregate the germline (Shirae-Kurabayashi and Nakamura, 2018; Takamura et al., 2002). Colonial botryllid ascidians are formed by genetically identical modules called zooids that are generated by budding and connect through a common vasculature. New zooids are continuously generated by the aggregation of hemoblasts, which are ASCs with germ/soma potential (Manni et al., 2019; Rinkevich et al., 2013; Rosental et al., 2018; Rosner et al., 2009; Sunanaga et al., 2010). Botryllid ascidians are so far the only chordates shown to clearly present adult PriSCs.



	Adult PriSCs	Regenerative abilities*	Asexual reproduction**	Adult GMP expression			
				G/S	Germ.	Soma	Reg.
Ctenophora	?	Whole body	✔	?	GSCs	ASCs	✔
Porifera ★	Archaeocytes Choanocytes	Whole body	✔	PriSCs	GSCs	ASCs	✔
Placozoa	?	Whole body	✔	?	?	?	?
Anthozoa	?	Whole body	✔	?	GSCs	?	?
Hydrozoa ★	i-cells	Whole body	✔	PriSCs	GSCs	ASCs	✔
Staurozoa	?	Whole body	✔	?	?	?	?
Scyphozoa	?	Whole body	✔	?	?	?	?
Cubozoa	?	Whole body	✔	?	?	?	?
Xenacoelomorpha ★	Neoblasts	Whole body	✔	PriSCs	GSCs	ASCs	✔
Nematoda	No	None/low	No	No	GSCs	No	No
Arthropoda	No	None/low	No	No	GSCs	No	No
Platyhelminthes ★	Neoblasts	Whole body	✔	PriSCs	GSCs	ASCs	✔
Annelida	MPGZ?	Whole body	✔	?	GSCs	ASCs	✔
Mollusca	?	High	No	?	GSCs	?	?
Hemichordata	?	Whole body	No	?	?	?	?
Echinodermata	?	Whole body	✔	?	GSCs	?	?
Cephalochordata	?	High	No	?	GSCs	ASCs	✔
Urochordata ★	Hemoblasts	Whole body	✔	PriSCs	GSCs	ASCs	✔
Vertebrata	No	None/low	No	No	GSCs	No	No

*Highest regenerative potential described within the phyla.
**Modes comprising fission, fragmentation and budding. At least one instance within the phyla.

Figure 5. Distribution of adult PriSCs, regeneration potential, asexual reproduction and GMP expression across Metazoa. Animal tree depicting the major animal phyla with a focus on relevant bilaterian and cnidarian lineages. Phyla comprising organisms with *bona fide* adult PriSCs are highlighted with a star symbol. The presence of adult PriSCs has been linked to whole-body regeneration and asexual reproduction (color coded in blue). Conversely, the absence of adult PriSCs has been associated with absent or low regenerative potential and the inability to reproduce asexually by fission, fragmentation or budding (color coded in orange). The last four columns inform about the adult contexts in which GMP marker genes (e.g. *vasa, pl10, piwi, tudor, nanos*) have been found enriched across the animal tree, including cells with mixed germ/soma potential (G/S), germline (germ), somatic lineages (soma) and regeneration (reg). Data sourced from Fierro-Constaín et al., 2017; Rinkevich et al., 2022; van Wolfswinkel, 2014.

During embryogenesis, echinoderms (e.g. sea urchins and sea stars) develop into free-swimming larvae consisting of somatic larval tissues and a set of cells, traditionally considered PGCs, that inherit germline potential and multipotency marker gene products (Perillo et al., 2021; Wessel et al., 2014). The mixed germ/soma potential of these cells, which constitute the foundation of the juvenile echinoderm (i.e. the adult rudiment), has however led to the alternative hypothesis that these constitute larval

PriSCs (Juliano et al., 2006; Juliano et al., 2010; Voronina et al., 2008). At adult stages of echinoderms, the presence of adult PriSCs has been hypothesized but direct evidence is lacking. While sea urchins present rather limited adult regenerative capacity, crinoids such as feather stars (e.g. *A. mediterranea*) can regenerate all appendices and internal organs, including gonads (Candia-Carnevali et al., 2009). Crinoids present both tissue-resident and circulating stem-like cells that contribute to regeneration and may consist of ASCs (Candia-Carnevali et al., 2009).

Acoel worms (e.g. *I. pulcra*, *H. miamia*) present an adult population of PriSCs similar to planarian neoblasts that underlie their ability to perform whole-body regeneration (Cannon et al., 2016; De Mulder et al., 2009; Hulett et al., 2022; Kimura et al., 2022; Srivastava, 2022; Srivastava et al., 2014). The phylogenetic position of acoels is unclear, with different lines of evidence placing them as a sister group either to all other bilaterians (Cannon et al., 2016) or to ambulacrarians (i.e. echinoderms and hemichordates) (Kapli and Telford, 2020; Mulhair et al., 2022; Philippe et al., 2019). As carriers of adult PriSCs, the resolution of acoels' position in the animal tree could have potential implications in our understanding of whether adult PriSCs constitute an ancestral trait in bilaterians.

In conclusion, within bilaterians, *bona fide* adult PriSCs have so far only been described in acoels, lophotrochozans (planarians) and urochordates (botryllid ascidians) **(Figure 5)**. In other highly regenerative bilaterians (e.g. annelids, feather stars), further studies are needed to elucidate the potency of their ASCs.

4.2.2. Adult PriSCs are found in non-bilaterian phyla

Non-bilaterian animals (i.e. sponges, ctenophores, placozoans and cnidarians) have in recent years attracted increasing scientific interest given their phylogenetic position as outgroups to bilaterians and their high body plasticity and regeneration potential. Efforts in characterizing their stem cell biology have shown that a common underlying factor for their plasticity and regenerative abilities is the presence of adult PriSCs that continuously segregate the germline and a diversity of somatic cell types throughout their lifetime (Rinkevich et al., 2022).

Sponges (Porifera) have been traditionally considered the sister group to all other animals due to their simple body plan and cell type composition. While their phylogenetic position in relation to the ctenophores is controversial (Borowiec et al., 2015; Redmond and McLysaght, 2021; Simion et al., 2017; Whelan et al., 2015), they are still key to understand the evolution of multicellular animals. Sponges can reproduce asexually by budding or fission. In addition, certain species inhabiting unstable environments have developed a mode of asexual reproduction called gemmulogenesis, in which different cell types including archeocytes aggregate and form a gemmule that can withstand adverse conditions until finding a favorable environment to hatch again (Ereskovsky, 2010). Sponges present two main stem cell types: choanocytes and archaeocytes. Archaeocytes are considered adult PriSCs, as they give rise to the germline and somatic cell types such as the choanocytes. Choanocytes are ciliated cells forming the feeding chambers that can dedifferentiate into archaeocytes or transdifferentiate into the germline (Ereskovsky, 2010; Funayama, 2013; Funayama et al., 2010). New studies have shown that GMP genes are not only expressed in germ cells, archaeocytes and choanocytes, but also in pinacocytes and vacuolar cells (Alié et al., 2015; Ereskovsky et al., 2015; Fierro-Constaín et al., 2017; Lavrov et al., 2018). This suggests that most of the sponge cell types may retain a certain level of multipotency and thus continuously adjust to the requirements of the organism for reproduction, growth, or regeneration.

Ctenophores, also called comb jellies, have recently been established as model organisms in evo-devo (e.g. *Mnemiopsis leidyi*, *Pleurobrachia pileus*) (Jager et al., 2011a; Pang et al., 2010). It is currently disputed if sponges or ctenophores form the phylogenetic sister group to all other Metazoa (Borowiec et al., 2015; Kapli and Telford, 2020; Simion et al., 2017; Whelan et al., 2017). Despite presenting capacities for whole-body regeneration, little is known about their stem cell biology (Edgar et al., 2021; Ramon-Mateu et al., 2019). Most ctenophore species reproduce sexually and are hermaphroditic, and so far only ctenophores of the order Platyctenida have been observed to reproduce asexually by fragmentation (Glynn et al., 2019). Characterizing the expression of conserved GMP factors like *nanos*, *piwi* and *vasa* in adult ctenophores revealed the location of the germline and of ASCs concentrating in defined

growth zones (e.g. tentacle root, aboral sensory complex) (Alié et al., 2011). GSCs have not been identified during embryonic or larval development, suggesting that germline segregation takes place at later stages by inductive mechanisms (Reitzel et al., 2016). So far, post-embryonic PriSCs have not been described in ctenophores, yet the potential of ASC populations remains unknown.

Placozoans are microscopic, free-living animals, with the simplest body plan among Metazoa. Their phylogenetic position remains unresolved, and only a handful of species have been identified, with *Trichoplax adhaerens* being the first and best studied (F. E. Schulze, 1883). *Trichoplax* reproduce asexually by fission or budding, and there is indirect evidence of sexual reproduction (Signorovitch et al., 2005). Interestingly, placozoans are the only animals to lack *vasa* and *piwi* orthologs, which have likely been lost in their lineage. Their reproductive strategies and regenerative abilities are only beginning to be understood, but so far their stem cell biology remains uncharacterized (Mayorova et al., 2021; Romanova et al., 2022; Signorovitch et al., 2005).

The phylum Cnidaria represents the sister group to all bilaterians. It comprises jellyfish, sea anemones and corals, among other organisms. Their remarkable plasticity, regenerative potential, and biological immortality has attracted an increasing interest in their stem cell biology. Yet, stem cells have so far only been characterized in hydrozoan species, which present pluri- or multipotent ASCs in the form of interstitial stem cells (i-cells). Yet, stem cells of non-hydrozoan cnidarians have so far remained elusive.

In summary, hydrozoan i-cells and sponge archaeocytes and choanocytes are the only currently confirmed adult PriSCs in non-bilaterian model organisms. These examples, together with the ones found in acoels, ascidians and flatworms, have led to question if ASCs with mixed germ/cell potential have evolved convergently or may be ancestral to all animals. However, the low diversity of taxa studied within each phyla hampers our understanding of stem cell evolution. In my work, I have investigated ASCs in the anthozoan cnidarian *Nematostella vectensis* with the objective of broadening our

understanding of stem cells among cnidarians and providing a steppingstone in the quest to elucidate cnidarian and animal stem cell evolution.

4.3. Cnidarians at the origin and forefront of stem cell research

Cnidarians are characterized by the shared presence of stinging cells called cnidocytes, a unique cell type involved in preying and defense. Cnidarians present a relatively simple body plan composed of an epidermis, an inner gastrodermis, and a single body opening that acts both as mouth and anus (Genikhovich and Technau, 2009b). Within Cnidaria, we find three main subphyla: (1) Anthozoa, which includes mostly sessile single or colonial polyps of the classes Octocorallia (soft corals, sea pens), Hexacorallia (stony corals and sea anemones) and Ceriantharia (tube-dwelling anemones); (2) Medusozoa, which includes mainly species with a free-swimming medusa stage in their life cycle, further subdivided into the classes Staurozoa, Hydrozoa, Cubozoa and Scyphozoa; and (3) Endocnidozoa, a newly identified clade that includes two groups of parasitic cnidarians, the Myxozoa and Polypodiozoa (Atkinson et al., 2018; DeBiasse et al., 2022).

The term “stem cell” was coined for the first time by August Weismann after investigating hydrozoan species, such as *Hydractinia echinata* and *Coryne pusilla* (Weismann, 1883). By researching these and other colonial hydroids, he described a population of histologically undifferentiated cells that were able to become germ cells, which led him to propose his germ plasm theory (Weismann, 1892). Nowadays, the interstitial stem cell lineage of hydrozoans is one of the best characterized stem cell systems among animals (Siebert et al., 2019; Varley et al., 2022). Cnidarians display remarkable regenerative abilities and body plasticity, which in the case of hydrozoans have been linked to the presence of multi- or pluripotent ASCs (i-cells). An extraordinary example of body plasticity is the hydrozoan jellyfish *Turritopsis dohrnii*, which can reverse its life cycle and is thus considered biologically immortal (Martell et al., 2016; Piraino et al., 1996). Given their relatively simple body plan, easy laboratory culture, genetic tractability and minimal ethical concerns, cnidarians are regarded as a key complement to vertebrate research in the quest to elucidate universal genes and molecular pathways involved in “stemness”. The characterization of the role

of adult cnidarian stem cells in regeneration and organismal immortality can potentially lead to key discoveries and applications in the field of regenerative biology (Bosch, 2008; Bosch, 2009; Frank et al., 2009).

4.3.1. Hydrozoan interstitial stem cells are multi- or pluripotent ASCs

Named after their location in-between epithelial cells in the ectoderm of hydroids, interstitial stem cells (i-cells) consist of a pool of self-renewing, undifferentiated, migratory cells that give rise to both germinal and somatic lineages throughout the lifetime of the organism (Frank et al., 2009). I-cells have been identified in a wide diversity of hydrozoan species, including jellyfish and siphonophores (Houliston et al., 2010; Seipel et al., 2004; Siebert et al., 2015), with the colonial marine hydroid *Hydractinia* (e.g. *H. echinata*, *H. symbiolongicarpus*) and the freshwater polyp *Hydra* (e.g. *H. oligactis*, *H. vulgaris*, *H. attenuata*, *H. magnipapillata*) as the best studied genera to date.

In *Hydra*, i-cell ablation and transplantation of single i-cells into stem cell-depleted hosts has repeatedly demonstrated the potential of i-cells to give rise to cnidocytes, sensory and ganglion neurons, gland cells and germ cells (Bosch and David, 1987; David and Murphy, 1977; David and Plotnick, 1980; Diehl and Burnett, 1964). **(Figure 6D).** In addition, there are two epithelial stem cell lineages, ectodermal and endodermal, which are composed of unipotent epithelio-muscular stem cells (Bode, 1996; Hemmrich et al., 2012) **(Figure 6C)**. *Hydra* i-cells concentrate in the middle part of the body column, from where they migrate and extend into the oral and aboral poles of the animal (head and foot) while differentiating **(Figure 6A, arrows**). Old cells are shed and replaced by new ones at the terminal regions of the animal. In addition, well fed polyps allocate new cells into buds that will generate a new animal through asexual reproduction. The generation of the different cell types needed in each region of the animal is achieved not only by constant repopulation from i-cells but also by a high phenotypic plasticity of differentiated cells that can transdifferentiate during their journey (Bode et al., 1986; Koizumi and Bode, 1991; Koizumi et al., 1988; Siebert et al., 2008). I-cell subpopulations with exclusive germinal fate when transplanted have

been identified in *Hydra* (Littlefield, 1985; Littlefield, 1991; Nishimiya-Fujisawa and Kobayashi, 2012; Nishimiya-Fujisawa C. and Sugiyama, 1993).

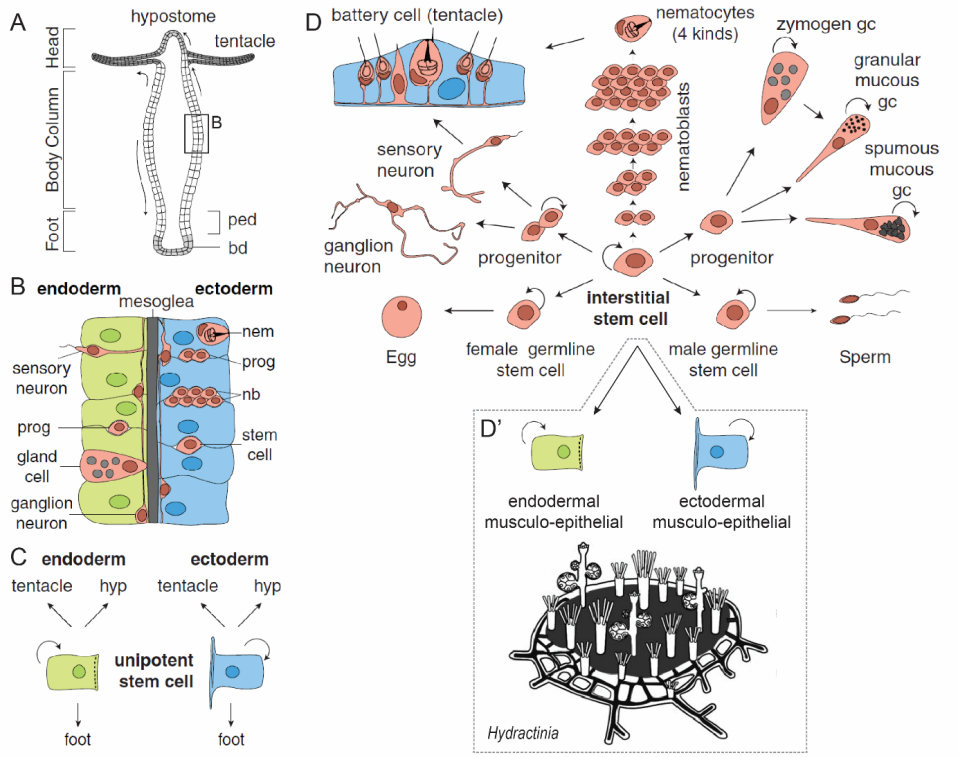


Figure 6. *Hydra* and *Hydractinia* stem cell lineages and their potential. (A-D) Schematics depicting the general morphology of the hydrozoan polyp body plan (A) and its ectodermal and endodermal cell lineages (B-D'). New cells are generated in the middle part of the polyp column (B, close-up of the box in A), which continuously displace old cells towards the oral and aboral poles of the polyp. *Hydra* presents one multipotent i-cell lineage (B-D, depicted in pink) and two unipotent endodermal and ectodermal epithelio-muscular lineages (B-D', depicted in green and blue, respectively). *Hydra* i-cells give rise to ganglion and sensory neurons, cnidocytes, gland cells (zymogen, mucous) and the germline (D). *Hydractinia* shares *Hydra* features but, in addition, its i-cells can also give rise to the epithelio-muscular lineages and are thus pluripotent (D'). ped: peduncle; bd: basal disk; gc: gland cell; nb: nematoblast; nem: differentiated nematocyte; prog: progenitor. Modified from Siebert et al. 2019, *Hydractinia* colony drawing from Müller and Leitz, 2002.

In *Hydractinia*, a single i-cell can give rise to all cell types, including endodermal and ectodermal musculo-epithelial cells (Künzel et al., 2010; Müller et al., 2004; Plickert et al., 2012; Varley et al., 2022). *Hydractinia* is so far the only organism in which a

single i-cell has been demonstrated to hold adult pluripotency (Varley et al., 2022). As many *Hydra* species, *Hydractinia* is dioecious and is the first cnidarian for which sexual chromosomes have been identified (Chen et al., 2022). It is also the only cnidarian in which post-embryonic germline segregation has been characterized at a molecular level. In this case, a single transcription factor, AP2, is sufficient to induce germ cell fate in i-cells. In addition, it is required for gonad formation in a non-autonomous way, which suggests that i-cells may be inducing somatic gonad formation in gonozooid polyps (DuBuc et al., 2020). A mammalian ortholog of AP2 plays a role in primordial germ cell induction in embryos, suggesting that it might be part of an ancestral program for germline induction (DuBuc et al., 2020; Magnúsdóttir et al., 2013).

In summary, hydrozoan species share a conserved population of multi- or pluripotent ASCs, the i-cells, which continuously segregate germinal and somatic lineages throughout the lifetime of the organism. The presence of such cells is tightly connected to the clonal and regenerative abilities, plasticity and biological immortality of hydrozoan cnidarians.

4.3.2. Stem cells of anthozoan, scyphozoan and staurozoan cnidarians

Knowledge on the stem cell biology of non-hydrozoan cnidarians is scarce, and so far, studies have failed to identify the equivalent to i-cells in non-hydrozoan model organisms (Gold and Jacobs, 2013; Technau and Steele, 2011). There is little to no data available on the stem cell biology of scyphozoan, cubozoan or staurozoan cnidarians, as their culture and maintenance in a laboratory setting has proven difficult. Ultrastructural studies have described amoeboid cells in anthozoan and scyphozoan species as potential stem cells (Gold and Jacobs, 2013), but no further evidence has yet been found.

Anthozoan cnidarians (e.g. corals and sea anemones) have attracted increasing interest in recent years for their ecological value in coral reefs, their symbiotic relationship with unicellular dinoflagellates and their phylogenetic position as sister group to all other cnidarians. Given that corals and sea anemones continuously produce millions of

gametes throughout their lifetime (which can extend up to hundreds of years in the case of corals), anthozoans are assumed to possess self-renewing adult GSCs. In corals, there is little molecular or cellular data available on stem cells or germline segregation. The best studied species is the reef-building scleractinian coral *Euphyllia ancora*, for which potential GSCs have been identified in the gastrodermal folds (i.e. mesenteries) (Shikina and Chang, 2016; Shikina et al., 2012; Shikina et al., 2015). In other scleractinian corals, such as *Acropora hyacinthus* and *Acropora palmata*, genome sequencing of different colony branches or sub-colonies have identified distinct, post-embryonic single nucleotide variants (SNVs) which are transmitted to the germline within the respective body parts (López-Nandam et al., 2021; Vasquez Kuntz et al., 2020). These data indicate that stony corals must present a population of ASCs that can give rise to both somatic and germinal lineages throughout the adult life of the coral colony. Yet, such cells have not been identified so far. In sea anemones, the stem cell biology of well-established models such as *Nematostella vectensis* and *Aiptasia pallida* remains largely unknown.

5. The sea anemone *Nematostella vectensis* as a stem cell research organism

In the past two decades, *Nematostella vectensis* has become a well-established anthozoan model organism in evolutionary and developmental research given its easy culture and maintenance in the laboratory and the accessibility to any life cycle stage, including sexual and asexual reproductive states (Genikhovich and Technau, 2009b; Layden et al., 2016; Reitzel et al., 2007). A combination of light and temperature stimuli is sufficient to induce synchronous spawning of males and females, which are able to produce numerous mature gametes every two to three weeks (Fritzenwanker and Technau, 2002; Genikhovich and Technau, 2009e; Hand and Uhlinger, 1992). The life cycle is relatively short, taking approximately three months using a daily feeding regime. Its sequenced genome (Putnam et al., 2007) has recently been upgraded to chromosome-level quality (Zimmermann et al., 2020). A great variety of molecular and genetic tools has been implemented in *Nematostella*, including *in situ* hybridization, immunostaining, S-phase labelling (Genikhovich and Technau, 2009a; Genikhovich and Technau, 2009c; Genikhovich and Technau, 2009d), morpholino and short-hairpin

RNA mediated knock-down (He et al., 2018; Magie et al., 2007; Rentzsch et al., 2008), transgenesis (Renfer et al. 2010) and CRISPR/Cas9 genome editing (Ikmi et al., 2014; Lebouvier et al., 2022).

5.1. Biology, reproduction and morphology

Nematostella is part of the family Edwardsiidae and can be found buried in the sediment of estuarine environments such as brackish water pools or salt marshes along the coasts of North America and England. In these quickly changing ecological spaces, it is able to withstand significant temperature and salinity fluctuations (Hand and Uhlinger, 1994).

Nematostella is dioecious, presenting male and female individuals that spawn sperm and eggs, respectively. Fertilization takes place externally and is followed by embryonic development, which presents a free-swimming larva called planula that settles and transforms into a primary polyp after approximately a week at 25°C. Upon feeding, primary polyps will go through a juvenile growth phase. After three months of growth, individuals start to spawn mature gametes and are then considered sexually mature adults. *Nematostella* polyps can also reproduce asexually by fission during both juvenile and adult stages (Fritzenwanker et al., 2007; Hand and Uhlinger, 1992).

As other cnidarians, *Nematostella* is considered diploblastic, presenting an outer epidermis and an inner gastrodermis separated by a thin layer of extracellular matrix called mesoglea (Shick, 1991). *Nematostella* displays a single oral opening that acts both as mouth and anus and that is surrounded by a crown of up to 18 tentacles involved in preying. Along the oral-aboral axis, its body is divided into three different regions: head (capitulum), body column (scapus) and foot (physa). The gastrodermis lines the gastric cavity and forms eight folds called mesenteries, which extend along the oral-to-aboral axis of the animal **(Figure 7B)** (Shick, 1991)**.** The gastrodermis is of endodermal embryonic origin except for the outer pharynx lining and the distal tip of the mesenteries (i.e. cnidoglandular tract), which have ectodermal embryonic origin (Shick, 1991; Steinmetz, 2019).

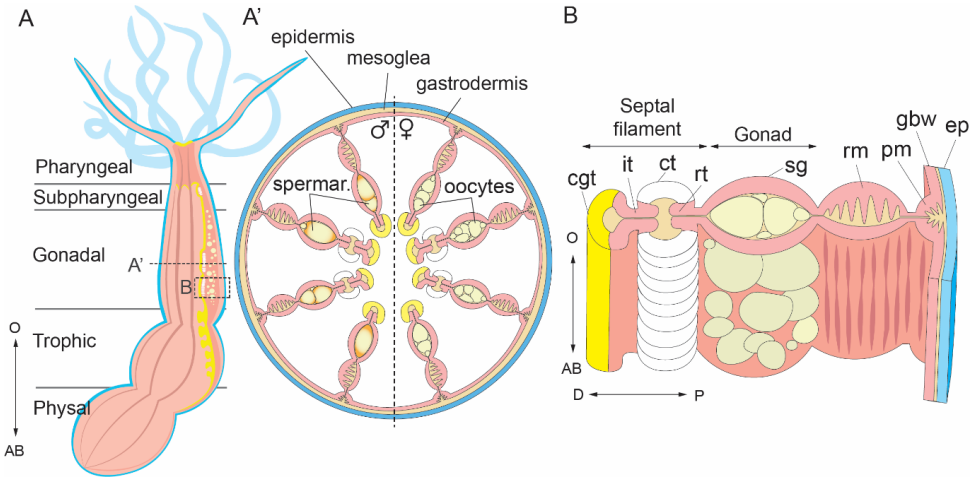


Figure 7. Morphology of the adult *Nematostella* polyp and its gonadal mesentery. (A, B) Schematics portraying a longitudinal section of an adult *Nematostella* (A) and a cross section of the body column comparing male and female gonadal mesenteries (A'). **(B)** 3D drawing of a piece of trilobed gonadal mesentery depicting the different tracts along the distal (D) to proximal (P) axis relative to the body wall. cgt: cnidoglandular tract; it: intermediate tract; ct: ciliated tract; rt: reticulate tract; sg: somatic gonad epithelium ; rm: retractor muscle; pm: parietal muscle: gbw: gastrodermal part of the body wall; ep: epidermis; spermar.: spermaries; O: oral; AB: aboral.

The morphology, function and cell type composition of the mesentery varies along the oral to aboral axis, but these different regions have not been formally named previously. In this manuscript, I refer to the different morphological regions of the mesentery as pharyngeal, subpharyngeal, gonadal, trophic and physal **(Figure 7A)**. Orally, mesenteries connect to the pharynx, an ectodermal epithelial ring that constitutes the oral opening of the animal. The subpharyngeal region of mesenteries is devoid of gametes and can be considered a transition zone between the pharynx and gonadal mesentery regions. The gonadal mesentery region harbors oocytes and spermaries of different sizes, and is followed aborally by the trophic region, which wraps around the prey during digestion (Shick, 1991; Steinmetz, 2019). Finally, the physal region of the mesentery shows a reduced size and complexity towards the aboral end of the body column **(Figure 7A)**. While *Nematostella* is considered to lack true organs, the mesentery is a complex tissue with specialized, longitudinal "tracts" that are enriched for different cell types and run along the oral-aboral axis **(Figure 7B)**

(Daly et al., 2003; Van-Praët, 1985). From proximal to distal, the first tract or region found in the mesentery is the parietal muscle at the base of the mesentery, which is in direct continuity with the body wall gastrodermis along the entire oral-aboral axis of the body column **(Figure 7B)** (Frank and Bleakney, 1976). The parietal muscle is followed by a thin section that joins the parietal and the retractor muscle. In the gonadal mesentery, the retractor muscle is followed by a gonad tract comprising a gonadal epithelium that includes vitellogenic nurse cells and mucus cells. Early oocytes are thought to emerge and bulge from the gonadal epithelium into the mesoglea, where they undergo oogenesis in an asynchronous way **(Figure 7B)** (Eckelbarger et al., 2008; Lebouvier et al., 2022; Moiseeva et al., 2017). Males resemble females in their morphology, differing in the presence of spermaries instead of oocytes in the gonad mesoglea **(Figure 7A')** (Tucker et al., 2011). The tip of the mesentery is called the septal filament **(Figure 7B)**, which comprises the reticulate tract (**Figure 7B, "rt"**) and the cnidoglandular tract **(Figure 7B, "cgt")** if unilobed, and also the ciliated **(Figure 7B, "ct")** and intermediate tracts **(Figure 7B, "it")** if trilobed (Daly et al., 2003; Malcolm Shick, 2012; Ruppert et al., 2004; Van-Praët, 1985). The pharynx and two pairs of primary mesenteries form during larval development and are thus already present in the primary polyp (Layden et al., 2016) Upon feeding, primary mesenteries start to grow and extend aborally, and during juvenile growth three more pairs of mesenteries develop (Jahnel et al., 2014). Around 3 months after fertilization, a gonad region emerges between the subpharyngeal and trophic regions of each mesentery **(Figure 7A)** (personal observation).

5.2. Stem cell biology in *Nematostella vectensis*: state-of-the-art

As other cnidarians, *Nematostella* can perform whole-body regeneration when bisected at any point of the body column, similarly to the process of asexual reproduction by fission (Amiel et al., 2015; Amiel et al., 2019; Layden et al., 2016). Recent work has demonstrated that mesenteries are indispensable for regeneration to take place, yet the underlying cellular or molecular mechanisms remain undescribed (Amiel et al., 2015; Amiel et al., 2019; Röttinger, 2021). In addition, *Nematostella* can withstand extremely long periods of starvation by shrinkage. This process can be reverted by re-feeding,

which causes explosive regrowth (K. Garschall, personal communication). These observations point to the presence of so far undescribed ASCs in the juvenile and adult mesenteries, which likely contribute to the regenerative abilities and body plasticity of *Nematostella*.

5.2.1. Potential PGCs are segregated at the end of larval development

So far, efforts on identifying and characterizing stem cells in *Nematostella* have focused on embryonic and larval stages. As successfully done for other metazoans, a common strategy applied to identify stem cells in *Nematostella* has been to characterize the expression and location of conserved GMP gene orthologs (e.g. *vasa*, *piwi*) in *Nematostella*.

Nematostella Vasa2 protein is present in germ granules of mature oocytes and is found ubiquitously at the blastula stage **(Figure 8)** (Chen et al., 2020; Praher et al., 2017). At the onset of gastrulation, the start of the embryonic expression of *vasa2* and other GMP genes (e.g. *piwi1, piwi2, tudor, vasa1*, *pl10*) takes place in the pre-endodermal plate(Chen et al., 2020; Extavour et al., 2005; Praher et al., 2017). Within the same time period, Vasa2 becomes gradually undetectable from the aboral towards the oral pole **(Figure 8)**. At planula stages, GMP markers are only present in the endoderm and are progressively restricted to two endodermal cell clusters at the basis of the pharynx in each of the primary mesenteries of the primary polyp **(Figure 8, red cells)** (Chen et al., 2020; Extavour et al., 2005; Praher et al., 2017). This process is regulated by the Hedgehog pathway, which in planula and primary polyp stages defines a boundary where the pharyngeal ectoderm (expressing the ligand *hh1*) and the endoderm (expressing the receptor *ptc*) meet (Chen et al., 2020). The expression of *nanos* orthologs during embryonic and larval development differs from the expression of other GMP genes. At the planula stage, *nanos2* expression is not only found in the endoderm but also in the pharyngeal ectoderm. At the primary polyp stage, the highest level of *nanos2* expression coincides with the mesenterial cell clusters expressing GMP genes, but is additionally found in single cells in the gastrodermis and pharynx (Extavour et al., 2005). *nanos1* is expressed in single cells in the gastrula and planula

ectoderm and in the developing endoderm corresponding to neural progenitors (Extavour et al., 2005; Steger et al., 2022).

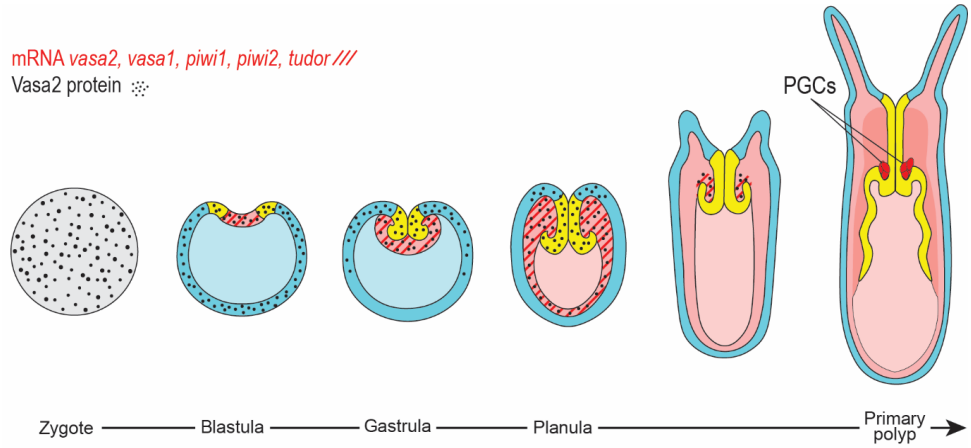


Figure 8. Determination of potential PGCs during embryonic and larval development in *Nematostella vectensis*. Depiction of the main stages of *Nematostella* embryonic and larval development highlighting the location of Vasa2 protein (black dots) and the regions co-expressing *vasa2*, *piwi1*, *piwi2* and *tudor* (red stripes). Data from Chen et al., 2020; Extavour et al., 2005; Praher et al., 2017. O: oral; A: aboral, 8d: 8 days after fertilization; PGCs: primordial germ cells.

Previous work proposed that the two GMP+ cell clusters determined at the end of the planula stage are PGCs and thus represent a case of embryonic germline segregation by epigenesis (Chen et al., 2020; Extavour et al., 2005) **(Figure 8)**. However, whether the presumed PGCs hold additional potentials beyond the germline remains yet to be addressed.

5.2.2. Adult stem cells remain elusive in *Nematostella*

In my work, I employ the term 'adult stem cell' (ASC) to refer to stem cells present at post-larval stages of *Nematostella* (i.e. juvenile and adult). As previously stated, few studies have investigated the stem cell biology of *Nematostella* beyond the primary polyp stage, and thus ASCs and their potential have remained uncharacterized.

Analysis of Vasa2 protein during juvenile growth and at adult stages revealed that, upon feeding, the presumed PGCs appear to proliferate and migrate into the developing mesenteries, where they give rise to the germline at sexual maturity (Chen et al., 2020).

Therefore, these Vasa2+ cells constitute potential adult GSCs in *Nematostella*. As the germline develops, GMP genes such as *piwi1*, *piwi2*, *vasa1* and *vasa2* continue to be expressed in *Nematostella* oocytes and in spermatogonia (Praher et al., 2017). Vasa2 protein has been detected in perinuclear germ granules similar to the *nuage* in growing oocytes and spermatogonia of *Nematostella* (Chen et al., 2020; Praher et al., 2017). While ultrastructural data suggests that early oocytes bulge into the mesoglea from the basis of the somatic gonad epithelium, the location of mitotic or meiotic oogonia remains uncharacterized (Eckelbarger et al., 2008; Tucker et al., 2011).

The stem cell source of adult somatic lineages, such as cnidocytes, neurons, gland cells, muscle cells or epidermal cells, remains as well unknown in *Nematostella.* So far, their developmental origins have only been investigated during embryonic and larval development (Richards and Rentzsch, 2014; Tournière et al., 2021). A recent scRNA-seq study that included juvenile and adult tissues has proposed differentiation trajectories for a neuroglandular lineage in *Nematostella* (Steger et al., 2022) similar to the one described *in Hydra* (Siebert et al., 2019). However, scRNA-seq studies in *Nematostella* have so far failed to unambiguously identify an ASC population upstream of the neuroglandular and cnidocyte progenitors (Sebé-Pedrós et al., 2018; Steger et al., 2022). Thus, the location of *Nematostella* ASCs and their role in homeostasis, growth and regeneration remain unknown.

6. Aims of the study

The present thesis intends to address the following questions:

- Are there adult stem cells in *Nematostella vectensis*?
- If so, what is their potential?
- Are there conserved features between anthozoan and hydrozoan adult stem cells?

The aim of my thesis is thus to characterize the location and potential of ASCs in *Nematostella vectensis.* To do so, I employed two different, complementary approaches, as reflected in the two papers presented.

Approach #1 (Paper I)

The main project of my thesis has been to characterize the location of potential stem cells and germline progenitors by studying GMP marker orthologs in juvenile and adult polyps. So far, GMP orthologs have been shown to be expressed in oocytes and spermatogonia of adults (e.g. *vasa1*, *vasa2*, *piwi1* and *piwi2*) (Chen et al., 2020; Praher et al., 2017). In addition, putative Vasa2+ germline stem cells have been described in the *Nematostella* mesenteries (Chen et al., 2020). Whether Vasa2+ cells hold a potential beyond the germline has not been addressed. Thus, the aims of this project were:

- To characterize the expression of a set of conserved GMP orthologs (*piwi1*, *piwi2*, *vasa1*, *vasa2*, *pl10*, *tudor*) in adult *Nematostella*.
- To characterize the location and colocalization of Vasa2 and Piwi1 proteins in juvenile and adult *Nematostella* by combining Vasa2 immunostaining and a Piwi1 knock-in fluorescent reporter line.
- To assess the potential of Vasa2+/Piwi1+ stem-like cells by *piwi1* and *vasa2* transgenic fluorescent reporter analysis in combination with a *soxB(2)* promotor-driven neuronal progenitor fluorescent reporter line.

Approach #2 (Paper II)

In a second approach, I aimed to identify potential stem-like cell populations in an unbiased manner by performing a single cell RNA-sequencing (scRNA-seq) experiment. Two whole-polyp scRNA-seq analyses including juvenile and adult stages have been previously published, neither of which could unambiguously identify clusters of stem-like cells (Sebé-Pedrós et al., 2018; Steger et al., 2022). Approach #1 has revealed the presence of a population of stem-like cells in the mesenteries of juvenile and adult *Nematostella* polyps, whose cell composition remains poorly described. As the mesenterial stem-like cell population may have been overlooked in previous scRNA-seq studies, I performed scRNA-seq of the adult female mesentery together with my colleague M. Lebouvier, in order to enrich for the stem-like cell-containing region of the mesentery (see approach #1). In a project co-authored with M.

Lebouvier, the overall aim was to produce a cell atlas of the whole adult female mesentery, including the stem-like cells. In this thesis, I will focus on the identification and characterization of adult stem cells in *Nematostella*. Thus, in the context of my thesis the specific aims of this approach were:

- To perform scRNA-seq of an adult female mesentery to identify potential adult stem and/or progenitor cell clusters (e.g. germline, neural).
- To place the mesenterial stem/progenitor cell clusters in a whole polyp context by combining our dataset with a published, whole polyp dataset (Sebé-Pedrós et al., 2018).
- Identify and characterize the expression of conserved and new marker genes that allow discerning between stem and progenitor cells.

Ultimately, by defining ASCs in *Nematostella*, I hope to contribute to our understanding of stem cell evolution within cnidarians and unveil the cellular basis of *Nematostella*'s striking body plasticity and regenerative abilities.

Chapter 2: Summary of the results

1. An adult stem-like cell population contributes to germinal and somatic lineages in the sea anemone *Nematostella vectensis* (Paper I)

Within cnidarians, the presence of adult stem cells (ASCs) holding both germinal and somatic potential has only been described in hydrozoan species and is thus so far considered a hydrozoan-specific trait (Technau and Steele, 2011). To identify potential ASCs in *Nematostella*, in Paper I I carefully characterized the mRNA expression and protein location of *piwi1* and *vasa2* genes in juvenile and adult stages. In addition, transgenic reporter lines were generated to trace their potential.

Characterization of a set of GMP gene orthologs in adult *Nematostella* gonadal mesenteries by *in situ* hybridization confirmed their expression in the germline (i.e. oocytes in females and spermatogonia in males) and revealed a population of small, basiepithelial cells expressing *piwi1*, *piwi2* and *tudor* in the extragonadal region of the septal filament, concentrating in the reticulate tract **(Paper I, Figures 1B-C' and S1)**. Vasa2 immunostaining in combination with a newly generated knock-in reporter line expressing mOr2-Piwi1 fusion protein was used to validate co-localization of Vasa2 and Piwi1 proteins in spermatogonia, perinuclear granules in growing oocytes, and in the previously described small, extragonadal cells in the reticulate tract of each mesentery **(Paper I, Figures 1E-H' and S2)**. S-phase labelling revealed that these Vasa2+/Piwi1+ cells can divide **(Paper I, Figures 1H-H' and S3)**. These Vasa2+/Piwi1+ cells correspond to the previously described Vasa2+ PGCs (Chen et al., 2020). In addition, I found Vasa2+/Piwi1+ cells not only in the gonadal regions of the adult mesenteries but all along the oral-aboral axis of the mesenteries, including pharyngeal, trophic and physal regions of both adult and juvenile polyps **(Paper I, Figures 2 and S4)**.

To investigate the potential of Vasa2+/Piwi1+ cells, I generated two new transgenic reporter lines for *vasa2* and *piwi1*: a promoter-driven *vasa2::mOr2* reporter line and a knock-in GFP-P2A-Piwi1 reporter line which generates separate GFP and Piwi1 protein products during translation. In combination with Vasa2 antibody and the *piwi1*^{mOr2} reporter line, I used those lines to perform fate mapping of Vasa2+/Piwi1+

mesentery cells. As expected, Vasa2 protein colocalized with high levels of *vasa2::*mOr2 in the germline and in Vasa2+/Piwi1+ extragonadal basiepithelial cells of the adult and juvenile mesenteries (**Paper I, Figures 3A-A''** and **S6B-D, F**). The same Vasa2+/Piwi1+ cells showed mOr2-Piwi1 protein colocalizing with high levels of GFP(-P2A-Piwi1) **(Paper I, Figure 3C-C'')**. In addition, lower levels of both *vasa2::*mOr2 and GFP(-P2A-Piwi1) were detected colocalizing in mOr2-Piwi1-/Vasa2- cells within the parietal muscle tract and gastrodermal body wall, thus likely labelling somatic progeny of Vasa2+/Piwi1+ cells **(Paper I, Figures 3B-B'', D-D'', F-F' and S11B-B'', F-F')**. These somatic progeny cells were abundant, often proliferative and basiepithelial, and distributed throughout the gastrodermis of juvenile and adult polyps **(Figures S6-S9)**. Thus, I concluded that Vasa2+/Piwi1+ cells constitute an adult stem-like cell population holding both germinal and somatic potential.

Remarkably, many of the somatic progeny cells that concentrated along the parietal muscle tract of each mesentery displayed migratory and neuronal shapes **(Paper I, Figures S6G-H'' and S9D', H, J-K)**. To test a potential neural identity of the somatic progeny, the GFP(-P2A-Piwi1) reporter line was crossed with the published neural progenitor reporter line *soxb(2)::mOr2* (Richards and Rentzsch, 2014). I showed that in these double reporter lines, low levels of GFP and mOr2 colocalized to basiepithelial, often proliferative cells in the muscle tracts and gastrodermal body wall **(Paper I, Figure 4B-D'')**. These results led me to propose that a set of gastrodermal neurons derives from a population of mesenterial Vasa2+/Piwi1+ stem-like cells holding germinal, neuronal and other somatic potentials **(Paper I, Figure 5)**.

2. Mesentery-enriched single-cell atlas of *Nematostella vectensis* uncovers complex cell composition and a stem-like cell population (Paper II)

While mesenteries are considered one of the most complex tissues of sea anemones, their cellular composition remains largely unexplored. In *Nematostella*, Vasa2+/Piwi1+ putative adult stem cells holding both germinal and somatic potential localize in a well-defined position (i.e. the reticulate tract) at the basis of the septal filament (see Paper I; **Paper II, Figure 1B-C**). Single-cell RNA-sequencing (scRNA-

seq) technology has allowed characterizing the cell diversity of the *Nematostella* polyp (Sebé-Pedrós et al., 2018; Steger et al., 2022). However, the complexity of the sexually mature mesentery has not been addressed, and adult stem cells have not been found in previous analyses. To address these limitations, I complemented here a previously published juvenile polyp scRNA-seq dataset (Sebé-Pedrós et al., 2018) with an adult female mesentery dataset comprised of microdissected mesentery samples, including the tissue where potential Vasa2+/Piwi1+ adult stem cells locate **(Paper II, Figure 1A)**. The result of this combined scRNA-seq dataset is an adult female mesentery cell atlas co-authored with M. Lebouvier (*in preparation*). In Paper II, I present a preliminary analysis of our dataset, with a focus on progenitor and stem-like cell types and states.

Applying a MARS-seq protocol and Seurat-based bioinformatic analysis (Jaitin et al., 2014; Satija et al., 2015), the transcriptomes of 6767 cells were grouped into 45 clusters **(Paper II, Figures 1D and S1)**. By analyzing the unique transcriptomic signature of each cluster and ISH patterns of respective marker genes, we assigned a putative cell identity to each cluster **(Paper II, Figure 1D)**. We validated our analysis by identifying clusters corresponding to previously described cell types such as cnidocyte and retractor muscle **(Paper II, Figure S2)**. We found a great diversity of endocytic, exocrine and neural cell types (Lebouvier, 2021) **(Paper II, Figure 1D)**. We also identified cell clusters connected to female sexual maturity such as vitellogenic somatic gonad cells (♀0_1) (Lebouvier, 2021) and oocytes (♀8) **(Paper II, Figure S4)**. In addition, our dataset highlights previously undescribed cell types of the septal filament, including ciliated tract cells (♀20) identified by the marker *zp2* **(Paper II, Figure 2F-G')** and proximal cnidoglandular tract cells (♀2_3) identified by the marker gene *semaphorin-5A* **(Paper II, Figure 2D-E)**. Interestingly, I found a cluster of *ki67*+ cells (♀36) consisting of cells identified by the marker gene *histone-H1 (v1g8097)* that locate to the proximal cnidoglandular tract of the trophic region and distal end of the ciliated tract **(Paper II, Figure 2J-N)**. Both clusters ♀2_3 and ♀36 were enriched for the stem cell/progenitor marker *nanos2* **(Paper II, Figure S5)** and partially shared their transcriptomic signature. Spatial expression analysis of cluster marker genes led me to propose that cluster ♀36 comprises putative proliferative progenitor cells of the ciliated

and cnidoglandular tracts, while ♀2_3 likely contains cnidoglandular tract cell precursors **(Paper II, Figure 2N)**. Finally, a cluster enriched for both germline/multipotency program (GMP) marker gene orthologs (e.g. *vasa*, *piwi*, *tudor*) and meiosis gene orthologs (e.g. *sycp1*, *hop1*) was identified (♀19) **(Paper II, Figure S5)**, likely comprising stem cells and meiotic oogonia.

To place our mesentery dataset within the context of a whole organism scRNA-seq dataset, we combined it with the published juvenile dataset (Sebé-Pedrós et al., 2018) and performed a new Seurat-based bioinformatic analysis, which yielded 78 cell clusters **(Paper II, Figure 3A)**. Most clusters were composed of cells originating from both datasets, indicating a low likelihood of batch effects **(Paper II, Figure S6A-B)**. In the merged dataset, co-enrichment of GMP marker genes was found in one single cluster (#8) **(Paper II, Figure 3B')**, which expressed *ki67* and other genes with putative roles in cell proliferation among its top marker genes **(Paper II, Figure 3B)**.

Subclustering of cluster 8 allowed identifying eight proliferative cell types or cell states **(Paper II, Figure 4A)**. Among those, I found that two subclusters with highest enrichment in GMP markers (#8.0 and #8.5) **(Paper II, Figures 5F and S7)** are highly similar in their transcriptomic signature except for the presence of meiosis genes in #8.5 **(Paper II, Figure 4C)**. This observation suggests that clusters #8.0 and #8.5 correspond to stem cells and meiotic oogonia, respectively. ISH revealed that specific marker genes for clusters #8.0 and #8.5 are expressed in reticulate tract cells of the female gonadal mesentery **(Paper II, Figure 4A-E')**, suggesting a close spatial proximity of stem cells and derived meiotic oogonia. The expression of marker genes for cluster #8.0 and GMP marker genes such as *vasa2* in reticulate tract cells led me to propose that cluster #8.0 corresponds to the population of putative Vasa2+/Piwi1+ adult stem cells described in Paper I **(Paper II, Figure 6)**. Other subclusters include a cluster of undetermined identity containing no specific marker genes (#8.2) and a cluster of putative cnidoglandular and ciliated tract progenitors (#8.7) **(Paper II, Figure 2J-N)**. In addition, I found a subcluster of cells enriched for mitotic spindle signature genes (#8.1) and subclusters of neuronal progenitor cells (#8.3, #8.4 and #8.6) that show an enrichment of neural transcription factor gene orthologs (e.g. *soxB(2)*,

achaete-scute and *otx*) and GMP markers (e.g. *nanos1*, *nanos2* and *pl10*) **(Paper II, Figures 4C and S7)**. The location of the subcluster #8.7 marker gene *histone-H1 (v1g8097)* to the proximal cnidoglandular tract of the trophic region and distal end of the ciliated tract coincides with a population of cells derived from Vasa2+/Piwi1+ adult stem-like cells highlighted by the *vasa2::mOr2* reporter line **(Paper I, Figure S8 C-C'', F-F', G''-G'''; Paper II, Figure 1C)**. This observation suggests that cluster #8.7 comprises cells derived from the Vasa2+/Piwi1+ adult stem cells represented by cluster #8.0 **(Paper II, Figure 6)**. The *vasa2::mOr2* and *piwi1*$^{P2A\text{-}GFP}$ reporter lines also revealed a population of *soxB(2)*+ NPCs in the mesenteries and body wall of the *Nematostella* polyp **(Paper I, Figure 4; Paper II, Figure 1C)**. I therefore proposed that the NPCs comprised within clusters #8.3, #8.5 and #8.6 also derive from the Vasa2+/Piwi1+ stem cells of cluster #8.0 **(Paper II, Figure 6)**.

Our mesentery-enriched polyp dataset thus allowed me to identify a cluster comprising proliferative progenitors and stem cells. Its subclustering led to the identification of a subcluster of Vasa2+/Piwi1+ adult stem-like cells and subclusters of germline (i.e. meiotic oogonia) and somatic progenitor cells (i.e. cnidoglandular and ciliated tract progenitors, NPCs) that are likely derived from Vasa2+/Piwi1+ stem-like cells. In conclusion, the scRNA-seq data presented in Paper II further supports the hypothesis presented in Paper I, which proposes a dual germ/soma potential for the population of adult Vasa2+/Piwi1+ stem-like cells in the mesenteries of *Nematostella*.

3. Additional results

A recent cross between *vasa2::mOr2* and the gastrodermal NPC reporter line *prdm14d::GFP* (Lemaître et al., 2022) has revealed colocalization between low levels of mOr2 and GFP (data not shown), suggesting that Prdm14d+ NPCs in the gastrodermis of *Nematostella* also derive from the Vasa2+/Piwi1+ stem-like cell population in the mesenteries. In the scRNA-seq dataset, *prdm14d (v1g96522)* is enriched in the NPC subcluster #8.3.

Chapter 3: Discussion

The objective of this thesis was to identify putative populations of adult stem cells (ASCs) and characterize their potential in the sea anemone *Nematostella vectensis*. Here, I provide the first evidence for a Vasa2+/Piwi1+ adult stem-like cell population holding both germinal and somatic potential in a non-hydrozoan cnidarian.

1. An adult stem-like cell population with germinal and somatic potential in *Nematostella*

1.1. Adult Vasa2+/Piwi1+ cells present conserved stem cell features

Stem cells are generally characterized by their abilities to self-renew and to give rise to progenitor cells that mitotically divide a limited number of times before differentiating into a given cell type. In addition, stem cells are often morphologically undifferentiated, with few exceptions such as *Hydra* epithelial stem cells (Bosch, 2007; Hobmayer et al., 2012). The preservation of their undifferentiated state and ability to self-renew are ensured by molecular determinants. More specifically, stem cells with germinal potential present highly conserved marker genes (germline/multipotency program, GMP) that protect their genome integrity (Juliano et al., 2010). In this thesis, I have described a population of cells that presents at least some of these features in *Nematostella*, and postulate that they constitute a population of ASCs with both germinal and somatic potential.

By characterizing GMP marker genes in juvenile and adult stages in *Nematostella*, I have identified a population of small cells in the mesenteries that express *piwi1*, *piwi2*, *tudor* and that colocalize Vasa2 and Piwi1 protein **(Paper I)**. These cells correspond to the presumed Vasa2+ PGCs previously described (Chen et al., 2020). Our scRNA-seq analysis **(Paper II)** has confirmed the presence of a single GMP+ ASC population in *Nematostella* enriched for the conserved GMP markers *piwi1*, *piwi2*, *piwi1-like*, *vasa1*, *vasa2*, *bol2*, *bol3* and *tudor*, supporting its stem cell identity and germinal potential. In addition, our scRNA-seq analysis has revealed new potential ASC markers that may play a role in stem cell and germ/soma potential maintenance, including

orthologs of the transcription factors *hlf* and *six-like*, as well as some genes without clear orthology (e.g. *v1g208008*).

Interestingly, our scRNA-seq data has shown that some GMP genes such as *nanos1*, *nanos2*, *pl10* and *pumilio* are not enriched in the ASC clusters, but in clusters of putative somatic progenitor cells (e.g. in neuronal progenitor cells) or differentiated cnidocyte, glandular and neuronal cells. These observations suggest a potential role for these genes in the specification and establishment of somatic lineages in *Nematostella*.

Using EdU labelling, I have shown that some Vasa2+/Piwi1+ cells undergo and complete S-phase **(Paper I)**. Interestingly, some Vasa2+/Piwi1+ cells had not incorporated EdU after relatively long pulses (3 days). This suggests that some of these cells are slow cyclers or quiescent, waiting for an external cue to re-enter the cell cycle (e.g. feeding or wounding). A way to investigate the percentage and location of slow cyclers within the Vasa2+/Piwi1+ cell population is to ablate fast cycling cells through irradiation or hydroxyurea treatments. The proliferative ability of Vasa2+/Piwi1+ cells was also supported by the scRNA-seq data, which shows enrichment of proliferation markers such as *ki67* and *cenpf* in the *vasa2+/piwi1+* stem-like cell subcluster **(Paper II)**. The ability of these cells to self-renew remains to be tested by single-cell transplant or genetic lineage tracing, which are currently not available for *Nematostella*.

Another general feature of stem cells is their ability to give rise to progenitor cells belonging to one or several cell lineages. By analyzing transgenic reporter lines for *piwi1* and *vasa2* **(Paper I)**, I have shown that the putative ASC population gives rise to abundant gastrodermal somatic cells, thus holding not only germinal but also somatic potential. As shown by short, 1-hour EdU pulses, a large proportion of the somatic progeny of Vasa2+/Piwi1+ cells is undergoing S-phase and thus likely consist of fast-cycling, proliferative progenitors. The presence of neural progenitor marker genes confirmed that a subset of the progeny of Vasa2+/Piwi1+ cells give rise to neural progenitors. Therefore, the dual germinal and neuronal potential shown in this work strongly suggests the presence of stem cells within the Vasa2+/Piwi1+ cell population.

1.2. The Vasa2+/Piwi1+ cell population consists of stem cells and oogonia

When addressing the level of heterogeneity of the Vasa2+/Piwi1+ cell population, two scenarios are possible: (1) the Vasa2+/Piwi1+ adult stem-like cell population is a homogeneous population with both germinal and somatic potential and without significant transcriptional differences between the cells; (2) it is a heterogeneous population consisting of different stem cell populations (e.g. germline, neural stem cells) and derived progenitor cells (e.g. germline progenitors, neural progenitors). The scRNA-seq data presented here **(Paper II)**, revealed two clusters enriched for *vasa2* and *piwi1* genes, which suggests a certain level of heterogeneity within the Vasa2+/Piwi1+ cell population. These two clusters present very similar transcriptomic profiles except for a few differentially expressed genes, including meiosis genes (e.g. *sycp1*, *hop1*) and some transcription factors (e.g. *hlf, six-like*). ISH on some of these differentially expressed marker genes revealed a similar expression pattern of cells along the basis of the septal filament of the gonadal mesentery (see **Paper II, Figure 5**), corresponding to the location of Vasa2+/Piwi1+ cells. Thus, according to my data, at least two subpopulations are found within the Vasa2+/Piwi1+ cell population, consisting presumably of (1) *hlf*+ stem-like cells with germ/soma potential and (2) *sycp1*+ pre-meiotic or meiotic germline progenitors (i.e. oogonia) that are potentially derived from the *hlf*+ stem-like cells **(Figure 9C)**. Our scRNA-seq data did not indicate a higher heterogeneity within these subpopulations. If present, it could be further resolved by scRNA-seq of FACS-sorted cells of *piwi1*^{mOr2} transgenic juvenile and adult polyps.

Another approach to address the heterogeneity within the Vasa2+/Piwi1+ cell population consists of following the lineage of single transplanted cells from a transgenic reporter line (e.g. *vasa2::mOr2* or *piwi1*$^{P2A\text{-}GPF}$) into wild-type hosts. This method would not only reveal the self-renewing ability of these cells but also clarify the diversity of their potentials. If, for instance, the progeny of a single cell develops only into somatic cell types, it could be concluded that somatic stem cells constitute a separate subpopulation of Vasa2+/Piwi1+ stem cells. In *Hydra*, for instance, i-cell transplants revealed a subpopulation of i-cells with restricted germinal potential

(Littlefield, 1985; Littlefield, 1991; Nishimiya-Fujisawa C. and Sugiyama, 1993)). Nevertheless, single cell transplant subjects the transferred cell to an artificial, stressful context that often leads to its death or affects its properties and potentials. These pitfalls need to be taken into consideration when interpreting the results of a single-cell transplantation experiment. Genetic lineage tracing tools such as the Brainbow system are powerful alternative methods to trace the potential of single Vasa2+/Piwi1+ cells (Livet et al., 2007; Weissman and Pan, 2015). For example, using the *hlf* gene as a driver in putative stem cells, the Brainbow system could reveal the diversity of potentials within the *hlf*+/Vasa2+/Piwi1+ stem-like cells. Another option for single-cell genetic lineage tracing is the use of photoconvertible reporter proteins. In my experience, however, non-invasive photoconversion of single Vasa2+/Piwi1+ cells is challenging due to their inaccessible location among highly auto-fluorescent structures deep within the gastrodermal folds of *Nematostella*. Ultimately, addressing the heterogeneity or homogeneity of a stem cell population is challenging, and it remains under scrutiny even in well-researched organisms such as planarians, whose neoblasts have been known for decades (Alessandra and Rossi, 2019; Molina and Cebrià, 2021; Zeng et al., 2018).

1.3. The progeny of the Vasa2+/Piwi1+ adult stem-like cell population comprises germinal and somatic lineages

1.3.1. The germinal progeny

The continuity of *vasa2* and *piwi1* gene and protein expression from the two stem cell clusters in the primary mesenteries of primary polyps to the adult mesenteries strongly suggests that the germline derives from mesenterial Vasa2+/Piwi1+ stem cells (Chen et al., 2020) **(Paper I)**. Our scRNA-seq data, in addition, shows that a subpopulation of Vasa2+/Piwi1+ cells express a high number of meiosis gene orthologs, thus likely consisting of premeiotic or meiotic oogonia **(Paper II)**. The expression of meiosis marker genes in cells located in the reticulated tract, where also Vasa2+/Piwi1+ cells are found, further supports that the germline derives from Vasa2+/Piwi1+ adult stem-like cells at the basis of the septal filament **(Figure 9B, C)**. Assessing the colocalization of meiosis markers with Vasa2 could confirm this assumption and assess the proportion

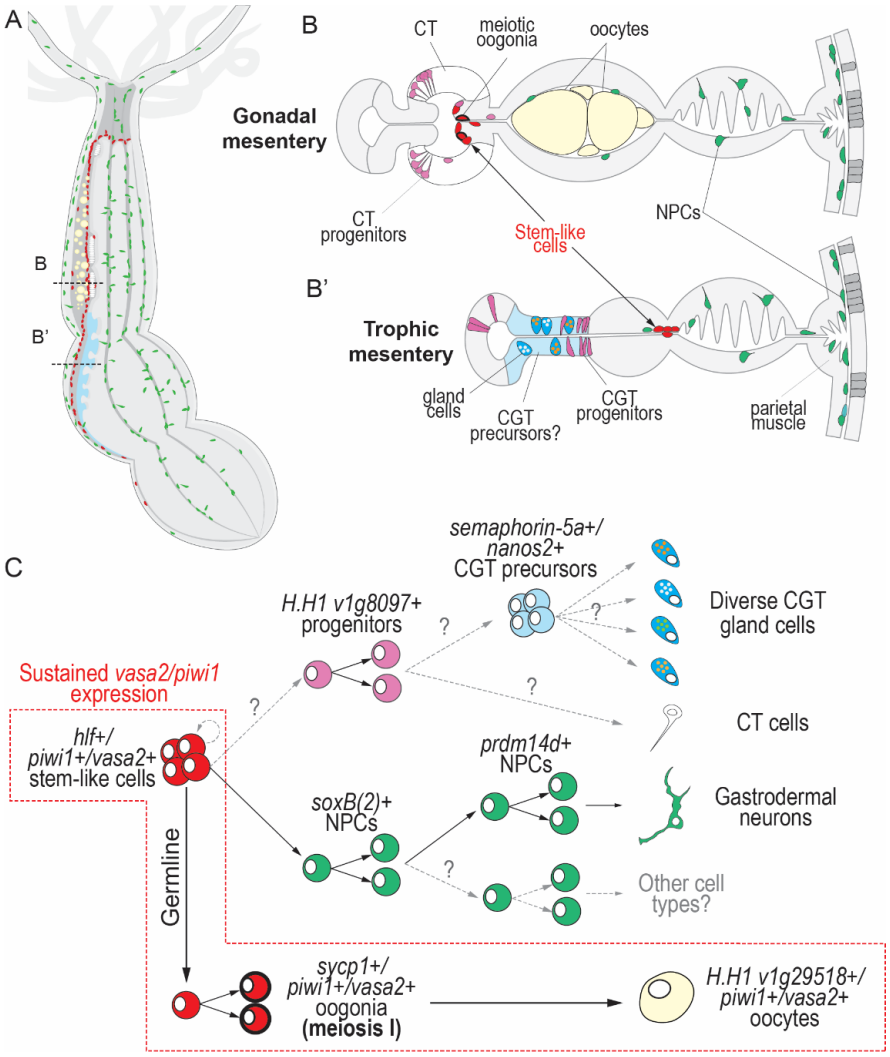


Figure 9. A population of Vasa2+/Piwi1+ adult stem-like cells gives rise to the germline and progenitor cells of neurons, cnidoglandular tract and ciliated tract. (A) Drawing of a longitudinal section of an adult female illustrating the mesenterial location of stem-like cells (red), oocytes (yellow), cnidoglandular tract precursors (light blue) and gastrodermal neuronal progenitors (green). **(B-B')** Illustrations of cross sections of the gonadal (B) and trophic (B') regions of the mesentery portraying the location of stem-like cells, proliferative CT, CGT and neuronal progenitors, gland cells and growing oocytes. **(C)** Schematic depicting the relationships between the cell populations depicted in (B), proposed after transgenic reporter line and scRNA-seq analyses presented in this thesis. Some hypothesized relationships require further experimental evidence to be confirmed (dashed arrows, question marks). CGT: cnidoglandular tract; CT: ciliated tract; NPCs: neuronal progenitor cells; H.H1*: histone-H1* gene.

of Vasa2+/Piwi1+ cells consisting of oogonia. The location where meiosis occurs remains to be assessed, for instance by detection of meiotic proteins (e.g. Sycp1) or meiosis-specific chromosomal arrangements (e.g. *bouquet*). In *Hydractinia*, a single transcription factor, AP2, is sufficient to induce germinal fate in i-cells (DuBuc et al., 2020). In our scRNA-seq analysis, the *Nematostella* AP2 ortholog is however not differentially expressed, and no expression was detected in adult gonadal mesenteries by ISH (data not shown). Thus, the molecular mechanisms inducing germline segregation and controlling the onset of meiosis gene expression in *Nematostella* remain unknown. The reporter lines described in this thesis will enable investigating the time and place of gametogenesis onset and the transition to sexual maturity in future experiments.

1.3.2. The somatic progeny comprises a diversity of progenitors

In **Paper I**, we have shown that *vasa2::mOr2* and *piwi1*$^{P2A\text{-}GFP}$ reporter lines highlight an abundant population of somatic progeny cells in the gastrodermis of *Nematostella* juveniles and adults. We have also observed that many of these cells undergo S-phase, which has led us to propose them as mitotically dividing progenitor cells (i.e. transit amplifying cells). Both transgenic reporter lines label putative progeny cells that concentrate along the parietal muscle of each mesentery and the body wall gastrodermis **(Paper I)**. These cells often display elongated shape and filopodia-like cell extensions, which suggests that they are migrating. Colocalization of *soxB(2)*::mOr2 with GFP(-P2A-Piwi1) **(Paper I)** and *vasa2::*mOr2 with *prdm14d::*GFP (data not shown) occurs in cells along the parietal muscle and gastrodermal body wall, which leads us to propose that a subset of the progeny cells consist of neural progenitor cells (NPCs) **(Figure 9A-B')**. In addition, *vasa2::*mOr2 labelled highly proliferative progenitor cells at the distal ends of the ciliated tract and at the proximal cnidoglandular tract of the trophic mesentery **(Paper I)**, which are likely represented in our scRNA-seq data within a *histone-h1(v1g8097)+* subcluster **(Paper II)**. The location of these *vasa2::*mOr2+ proliferative progenitors, together with shared marker genes with cnidoglandular clusters according to our scRNA-seq analysis, leads us to propose that they give rise to cells composing this tract such as

gland cells **(Figure 9B', C)**. The presence of *vasa2::*mOr2+ proliferative cells at the distal end of the ciliated tract suggests that these likely consist of ciliated tract progenitors **(Figure 9B, C)**. Combining *vasa2::*mOr2 immunostaining with ISH for gland cell or ciliated tract marker genes could confirm these assumptions. In addition, scRNA-seq of FACS-sorted *vasa2::*mOr2+ cells could inform of a potential gland cell or ciliated tract fate within the somatic progeny of the Vasa2+/Piwi1+ adult stem-like cell population and further resolve the differentiation trajectories.

1.3.3. Vasa2+/Piwi1+ stem-like cells at the origin of post-larval neurogenesis

In *Nematostella*, neurogenesis has been mainly characterized during embryonic and larval development until the primary polyp stage. During embryonic and larval development, SoxB(2)+ neural progenitors give rise to cnidocytes, ganglion neurons, and a neuroglandular lineage of sensory-secretory neurons, gland cells and other neural cell types (Nakanishi et al., 2012; Richards and Rentzsch, 2014; Steger et al., 2022; Tournière et al., 2022). A subpopulation of SoxB(2)+ neural progenitors that are Prdm14d+ generate gastrodermal neurons that concentrate along the parietal muscle and in the body wall gastrodermis (Lemaître et al., 2022). Here, I have characterized the *soxB(2)::*mOr2 line in juveniles and adults, finding high levels of mOr2 in gastrodermal and ectodermal cells with differentiated neuronal shapes (e.g. sensory cilia, long axons) that concentrate along the parietal muscle region **(Paper I)**. In addition, I have shown colocalization of low levels of *soxB(2)::*mOr2 protein and GFP(-P2A-Piwi1) protein in proliferating cells of the parietal and retractor muscle regions and body wall gastrodermis **(Figure 9A-B')**. These findings, together with the colocalization of *vasa2::*mOr2 and *prdm14d::*GFP in cells along the parietal muscle and gastrodermal body wall (data not shown) has led me to postulate that a subset of the progeny of Vasa2+/Piwi1+ stem-like cells consists of neural progenitor cells **(Figure 9C)**. This is also supported by our scRNA-seq data, which shows that within a single cluster enriched for proliferation markers (*ki67*+) and GMP genes we find a subpopulation of Vasa2+/Piwi1+ stem-like cells and three subpopulations enriched for *soxB(2)*, *prdm14d* and other neuronal transcription factor orthologs **(Paper II)**.

In this work, I present that Vasa2+/Piwi1+ adult stem-like cells in the mesenteries are a potential cellular source for SoxB(2)-driven neurogenesis in juvenile and adults. Due to the cytoplasmic inheritance and stability of the mOr2 protein in the *soxB(2)::*mOr2 reporter line, it is difficult to discriminate between progenitor cells and differentiating neurons. I can therefore not confidently identify all SoxB(2)+ progenitor cells and assess if they completely colocalize with GFP(-P2A-Piwi1)+ cells. Thus, it remains unknown whether all or just a subset of SoxB(2)+ progenitor cells derive from Vasa2+/Piwi1+ cells. A way to test if the Vasa2+/Piwi1+ stem-like cell population is the sole origin for SoxB(2)-driven neurogenesis in juveniles and adults would be to characterize the colocalization of SoxB(2) protein with GFP(-P2A-Piwi1) and *vasa2::*mOr2 by generating a SoxB(2) antibody or an endogenous SoxB(2) fusion protein reporter line.

It remains to be addressed whether the juvenile and adult SoxB(2)+ NPCs described here give rise to a similar array of cell types as during embryonic and larval development (e.g. cnidocytes, sensory neurons, gland cells, ganglion neurons) (Richards and Rentzsch, 2014; Steger et al., 2022; Tournière et al., 2022). A recent scRNA-seq data analysis that included all life stages of *Nematostella* has proposed that neurogenesis in juveniles and adults derives from a population of *soxC+/soxB(2)+* NPCs that follows the same trajectories as during embryonic and larval neurogenesis (Steger et al., 2022). Yet, evidence from lineage tracing experiments is missing and the stem cell population upstream of *soxC+/soxB(2)+* NPCs remains uncharacterized (Steger et al., 2022).

In this work, I propose that Vasa2+/Piwi1+ stem-like cells in the mesenteries give rise to SoxB(2)+ NPCs in juveniles and adults. Comparing my findings to published data on the protein and gene expression dynamics of *piwi1*, *vasa2* and *soxB(2)* genes during *Nematostella* embryonic and larval development reveals striking similarities that indicate that neurons might originate from undifferentiated, Vasa2+ cells during also during embryonic and larval neurogenesis **(Figure 10A)** (Chen et al., 2020; Extavour et al., 2005; Praher et al., 2017; Richards and Rentzsch, 2014). At blastula stages, Vasa2 protein is present ubiquitously (Chen et al., 2020; Praher et al., 2017), while

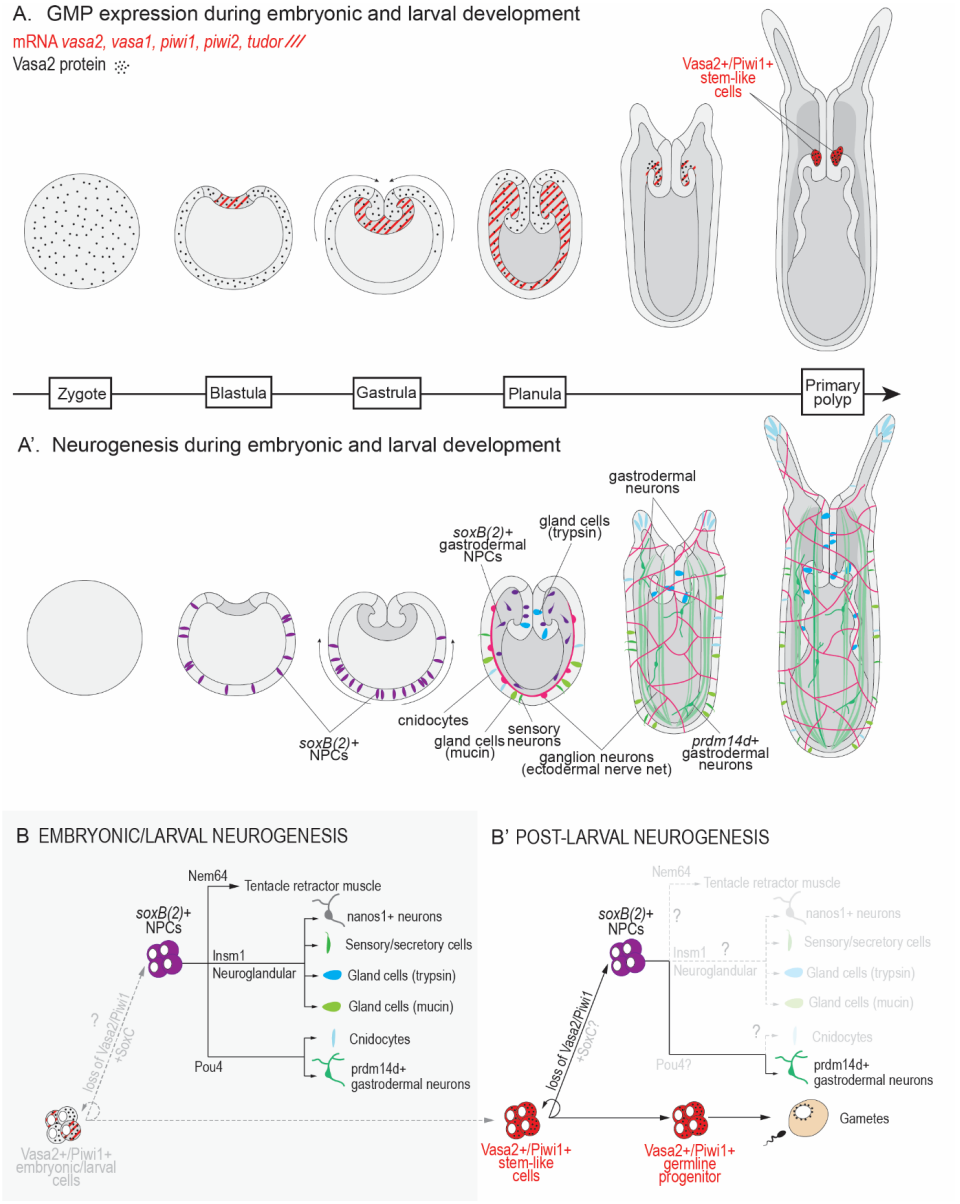


Figure 10. Hypothesis: undifferentiated Vasa2+ cells as a common origin for embryonic, larval and post-larval neurogenesis. (A, A') Schematics of longitudinal cross sections of developmental stages depicting published data on the location of Vasa2 protein (A, black dots), co-expression of GMP marker genes *vasa2*, *vasa1*, *piwi1*, *piwi2* and *tudor* (A, red stripes or red cells), the location of *soxB(2)*+ neural progenitor cells (A', purple cells) and the diversity of their progeny (A'). **(B, B')** Diagrams portraying undifferentiated Vasa2+ cells as an hypothetical origin for embryonic, larval and post-larval *soxB(2)*-driven neurogenesis. A subset of larval endodermal cells retains *vasa2+/piwi1*+ expression and become adult stem-

like cells (Chen et al., 2020), which may recapitulate embryonic and larval *soxB(2)*-driven neurogenesis. Data in (A, A') from Chen et al., 2020; Extavour et al., 2005; Lemaître et al., 2022; Praher et al., 2017; Richards and Rentzsch, 2014; Tournière et al., 2020 and Tournière et al., 2022. SoxC/SoxB(2)-derived lineages in (B, B') as proposed in Steger et al., 2022. Relationships between cell populations in (B, B') supported by experimental evidence outlined in black; hypothetical relationships depicted by dashed, grey arrows and highlighted by question marks.

vasa2 and *piwi1* mRNAs are not detected (Extavour et al., 2005) and a few cells start to express *soxB(2)* (Richards and Rentzsch, 2014) **(Figure 10A-A')**. At the onset of gastrulation, embryonic expression of *piwi1*, *vasa2* and other GMP genes is found orally in the presumptive endoderm and pharyngeal ectoderm (Extavour et al., 2005) **(Figure 10A)**. During gastrulation, Vasa2 protein is gradually lost from the aboral ectoderm, while it is enriched in the invaginating endoderm (Chen et al., 2020; Praher et al., 2017) **(Figure 10A)**. Complementary to Vasa2 protein loss, an abundance of *soxB(2)*+ cells is detected in the aboral half of the ectoderm, where neurogenesis of sensory neurons, gland cells and cnidocytes takes place (Nakanishi et al., 2012; Richards and Rentzsch, 2014; Tournière et al., 2022) **(Figure 10A')**. During larval development, *vasa2* and *piwi1* expression is gradually restricted to the endoderm and to two clusters of cells at the boundary with the pharyngeal ectoderm in the primary polyp (Chen et al., 2020) **(Figure 10A)**. At the same time, *soxB(2)*+ progenitors and their *insm-1*+ and *prdm14d*+ progeny appear in the endoderm (Lemaître et al., 2022; Richards and Rentzsch, 2014; Tournière et al., 2022) **(Figure 10A')**. Thus, the gastrula and larval stages display a wave of GMP gene expression loss from the ectoderm and the gastrodermis that results in their restricted expression in two gastrodermal clusters of Vasa2+/Piwi1+ cells. This dynamic loss of GMP gene expression seems to be followed by a complementary wave of *soxB(2)*-driven neurogenesis. These observations lead me to propose that Vasa2, and potentially other factors, may keep embryonic and larval cells in a multipotent, undifferentiated state that is abandoned once Vasa2 and GMP expression are lost and *soxB(2)*-driven neurogenesis ensues **(Figure 10B)**. My findings suggest that a similar process takes place at post-larval stages. In juveniles and adults, neural progenitor cells derived from Vasa2+/Piwi1+ cells likely lose Vasa2 and Piwi1 expression and start expressing *soxB(2)* **(Paper I)** while acquiring neural fate and giving rise to gastrodermal neurons (e.g. *prdm14d*+

neurons) **(Figure 10B')**. Neural progenitor cells derived from Vasa2+/Piwi1+ stem-like cells at post-larval stages may thus recapitulate embryonic and larval neurogenesis pathways **(Figure 10B')**, as suggested by trajectory reconstructions based on scRNA-seq analysis (Steger et al., 2022). The combined analysis of *piwi1*^{mOr2}, *piwi1*$^{P2A\text{-}GFP}$, *vasa2::mOr2* and *soxB(2)::mOr2* reporter lines during embryonic and larval development could test if GMP gene expression declines complementary to the progression of neurogenesis on a cellular level. In conclusion, Vasa2+ cells may be a common origin of neurons at embryonic, larval and post-larval stages in *Nematostella*. These Vasa2+ cells would correspond to the theorized primordial stem cells (PriSC), which are characterized by the presence of GMP marker genes such as *vasa* and *piwi*, and by holding both germinal and somatic potential (Solana, 2013).

1.3.4. The source of epidermal cells in *Nematostella*

Remarkably, all the reporter lines presented in this work (i.e. *piwi1*^{mOr2}, *piwi1*$^{P2A\text{-}GFP}$ and *vasa2::mOr2*) highlighted cells in the epidermis of *Nematostella* juvenile and adult polyps **(Paper I, Figure S9)**. This was surprising given that neither *piwi1* nor *vasa2* mRNA or protein could be detected in the epidermis. The presence of the fusion protein mOr2-Piwi1 in the epidermis reveals active expression of *piwi1* in these cells, suggesting that *piwi1* and *vasa2* mRNA levels may be below the ISH detection threshold. A more in-depth characterization of *GFP* and *mOr2* expression by ISH in transgenic reporter juvenile and adult polyps could further inform us of the logic behind the epidermal signal. Whether the epidermis constitutes a separate stem cell lineage, independent from the mesenterial Vasa2+/Piwi1+ adult stem-like cells, remains to be resolved. This question could be addressed by generating a reporter line for *hlf*, which based on our scRNA-seq analysis is highly specific for the Vasa2+/Piwi1+ stem-like cell subpopulation, to study if reporter protein is cytoplasmically inherited by epidermal cells. Ultimately, transplantation of transgenic mesentery of *piwi1*$^{P2A\text{-}GFP}$ and *vasa2::mOr2* polyps into a wild-type host and subsequent analysis of the epidermis could reveal if mesenterial Vasa2+/Piwi1+ stem-like cells constitute a cell source for the epidermis.

1.4. Potential roles during growth, homeostasis and regeneration

As other cnidarians, *Nematostella* displays high body plasticity. It grows and shrinks in response to food availability (K. Garschall, in preparation) or performs whole-body regeneration from buds or amputated sections of the body column. Adult stem cells are key sources for growth and regeneration processes in other similarly plastic animals such as hydrozoans (*Hydra*, *Hydractinia*), acoels or planarians (Bosch, 2007; Gahan et al., 2016; Gehrke and Srivastava, 2016; Srivastava, 2022; Wagner et al., 2011). Thus, it is likely that Vasa2+/Piwi1+ stem-like cells are one of the key cell populations underlying body plasticity in *Nematostella*.

Founder cells of the Vasa2+/Piwi1+ adult stem-like cell population have been described to proliferate and migrate into the emerging mesenteries of young juvenile polyps (Chen et al., 2020). I have shown that Vasa2+/Piwi1+ cells are present along the entirety of juvenile and adult mesenteries **(Paper I)**, suggesting that their presence is required broadly. In addition, the abundance of somatic progeny from Vasa2+/Piwi1+ cells in the juvenile gastrodermis **(Paper I, Figure S7A'')** has led me to propose that the Vasa2+/Piwi1+ adult stem-like cell population contributes significantly to juvenile growth by continuously generating new cells (e.g. neurons and other somatic cell types). The ability of *Nematostella* to shrink its body size while starving and to explosively regrow when food is available (K. Garschall, *in preparation*) may also rely on Vasa2+/Piwi1+ stem-like cells and their progeny. The response of Vasa2+/Piwi1+ adult stem-like cells to feeding and starvation is currently being investigated by colleagues.

To test the role of Vasa+/Piwi1+ stem-like cells during growth and germline segregation, I am analyzing *Nematostella vasa2* mutant animals generated by C-Y. Chen (Matt Gibson's lab, Kansas City, USA). Preliminary data suggests that *vasa2*-/- animals lack Vasa2+/Piwi1+ PriSCs and are unable to grow beyond the primary polyp stage after feeding (C-Y. Chen, personal communication). If validated, this observation would further confirm that Vasa2+/Piwi1+ cells in primary polyps do not consist of PGCs with restricted germinal potential but of PriSCs necessary for generating somatic lineages and essential for the initiation of juvenile growth.

Nematostella is used as research organism to understand animal whole-body regeneration (Amiel et al., 2015; Layden et al., 2016; Passamaneck and Martindale, 2012; Reitzel et al., 2007; Röttinger, 2021). It has been shown that populations of fast and slowly cycling, potential stem cells of unknown cellular identity play key roles during regeneration (Amiel et al., 2019). Interestingly, transplantation experiments have shown that a combination of mesentery and body wall tissue is sufficient for regeneration to take place, while these tissues fail to regenerate separately (Amiel et al., 2019). In addition, the presence of mesentery tissue is required for cell proliferation to take place in the regenerates, and thus the mesentery has been proposed as a potential source of signals and/or cells needed for regeneration (Amiel et al., 2019). Accordingly, the Vasa2+/Piwi1+ stem-like cells and progeny described in this work may constitute the hypothesized mesenterial cells required for whole-body regeneration as in *Nematostella*. This assumption remains to be tested, for instance by the analysis of *vasa2* and *piwi1* transgenic reporter polyps after mesentery and body wall grafting experiments, body column bisection or tentacle amputation. Asexual reproduction by transverse fission (i.e. physal pinching) resembles the process of regeneration, and it would therefore also be interesting to study *vasa2* and *piwi1* transgenic reporter lines during bud regeneration.

In bisected primary polyps, aboral portions lacking the two clusters of Vasa2+/Piwi1+ founder stem-like cells were able to completely regenerate pharynx and tentacles 8 days after amputation, while Vasa2+ cells were not restored (Chen et al., 2020). While this observation suggests that Vasa2+/Piwi1+ cells may not be needed during the regeneration of primary polyps, it may be a unique property of animals at this stage. In addition, it is unknown if Vasa2+ cells were eventually restored in the aboral regenerates, and if the resulting animals were viable and fertile.

In conclusion, mesenterial Vasa2+/Piwi1+ stem-like cells and their progeny are likely key players during growth and regeneration in *Nematostella*. This assumption will be tested in future studies.

2. Adult stem cells with germ/soma potential are likely an ancestral trait of cnidarians

2.1. Adult stem cells are poorly characterized in other anthozoan cnidarians

There is scarce molecular data about ASCs in anthozoans (e.g. corals, sea anemones, sea pens), with the stony coral *Euphyllia ancora* (Hexacorallia) as the best characterized species to date. Contrary to *Nematostella*, *E. ancora* presents a yearly gametogenic cycle, displaying a single round of synchronous gametogenesis per year. This allows to easily trace gametogenesis from its onset to the spawning of all mature gametes (Shikina et al., 2012). In this species, Vasa2 and Piwi1 immunostaining has revealed a population of small Vasa2+/Piwi1+ cells, proposed as germline stem cells, locating to the mesenteries of male and female individuals throughout the gametogenic cycle (Shikina et al., 2015). In light of the results presented in this thesis, I hypothesize that Vasa2+/Piwi1+ cells in *E. ancora* may hold not only germinal but also somatic potential. Remarkably, Vasa2+/Piwi1+ cells localize between the basis of the septal filament and the retractor muscle in *E. ancora*, corresponding to the location where Vasa2+/Piwi1+ cells are found in *Nematostella*. Thus, I postulate that the location of Vasa2+/Piwi1+ adult stem-like cells in this defined region of the mesenteries may be a conserved feature of hexacorallians. It would also be interesting to explore whether ASCs are also found within the so far uncharacterized gastrodermal folds of of octocoral, sycphozoan, cubozoan and staurozoan polyps.

Recent genomic studies have provided strong evidence supporting the presence of stem cells with both germinal and somatic potential in adult stony coral colonies by showing that single nucleotide variants (SNV) specific to different branches of a coral colony are transferred to the germline (López-Nandam et al., 2021; Vasquez Kuntz et al., 2020). Finding the same SNVs acquired during adult growth in both somatic tissues and gametes demonstrates that they must be derived from a common adult stem cell population (López-Nandam et al., 2021).

In conclusion, while ASCs remain poorly characterized in other anthozoan cnidarians, the available data supports the presence across hexacorallians of ASCs with dual

germ/soma potential expressing GMP marker genes such as *vasa* and *piwi* orthologs, likely locating to the mesenteries.

2.2. Vasa2+/Piwi1+ stem-like cells in *Nematostella* are likely homologous to hydrozoan interstitial stem cells

Interstitial stem cells (i.e. i-cells) have so far been considered a hydrozoan-specific trait as comparable stem cells have not been found in any non-hydrozoan cnidarians (Technau and Steele, 2011). I-cells have been characterized in a few hydrozoan jellyfish species (Carnea, 2004; Fujita et al., 2022; Leclère et al., 2012), but most knowledge stems from studies on exclusively sessile hydrozoan polyps such as *Hydra* and *Hydractinia.*

In this thesis, I have shown several cellular features of *Nematostella* Vasa2+/Piwi1+ stem-like cells that resemble those of hydrozoan i-cells. They both exhibit a small cell size, a high nucleus to cytoplasm ratio and their small numbers throughout the polyp (Frank et al., 2009). I-cells constitute about 4% of all cells in a *Hydra* polyp (David and MacWilliams, 1978). Preliminary flow cytometry data analyzing the proportion of mOr-Piwi1+ cells in juvenile *piwi1*^{mOr2} polyps shows that Vasa2+/Piwi1+ stem-like cells represent approx. 0,34% of all cells in a *Nematostella* juvenile (E. Pascual, unpublished), thus representing <10% of the abundance of *Hydra* i-cells.

Both *Hydra* i-cells and *Nematostella* Vasa2+/Piwi1+ cells are found in interstitial and/or basiepithelial locations. However, while Vasa2+/Piwi1+ cells in *Nematostella* locate within the gastrodermal folds **(Paper I)**, i-cells reside within the epidermis of hydrozoans. When it comes to embryonic development, *Hydractinia* i-cells arise in the endoderm and migrate into the epidermis at the end of the larval stage, when settlement takes place (Rebscher et al., 2008). Thus, both i-cells and Vasa2+/Piwi1+ stem-like cells appear to share a developmental origin in the endoderm (Chen et al., 2020; Extavour et al., 2005).

While i-cells concentrate in certain regions of the organism (e.g. middle band of the polyp epidermis, tentacle bulb in jellyfish), they are in comparison more broadly distributed than *Nematostella* Vasa2+/Piwi1+ cells, which locate to a precisely defined

region within the mesenteries. The existence of a niche has not been described outside of Bilateria, and, interestingly, such microenvironments appear to be absent in animals presenting ASCs with germ/soma potential (e.g. *Hydra*, planarians) (Bosch et al., 2010; Martinez et al., 2022; Rossi and Salvetti, 2019). Whether a niche-like, regulative microenvironment is present in the defined mesenterial region where the Vasa2+/Piwi1+ stem-like cells locate in *Nematostella* remains to be explored.

I-cells and Vasa2+/Piwi1+ stem-like cells in *Nematostella* also share the enrichment of GMP genes such as *piwi* and *vasa* (Leclère et al., 2012; Lim et al., 2014; Mochizuki et al., 2001; Rebscher et al., 2008). Other additional i-cell marker genes, such as *nanos* and *myc* gene orthologs (Mochizuki et al., 2000; Plickert et al., 2012), are rather expressed in somatic progenitor cell states in *Nematostella* according to our scRNA-seq data **(Paper II)**. Comparing the scRNA-seq transcriptomic signature of *Hydra* i-cells (Siebert et al., 2019) and *Nematostella* Vasa2+/Piwi1+ stem-like cells **(Paper II)** could further inform of shared genes and potentially find cnidarian-specific stem cell genes.

We have shown that the Vasa2+/Piwi1+ adult stem-like cell population in *Nematostella* presents both germinal and neural potential **(Paper I)**, a feature shared with hydrozoan i-cells (David, 2012; Müller et al., 2004; Siebert et al., 2019). I-cell-based neurogenesis is driven by *soxC* and *soxB* orthologs, and involves neuroglandular progenitor cells (Chrysostomou et al., 2022; Flici et al., 2017; Jager et al., 2011b; Siebert et al., 2019). These features are also found during *Nematostella* embryonic and larval development and supposedly also at post-larval stages (Richards and Rentzsch, 2014; Steger et al., 2022; Tournière et al., 2022), which suggests that adult neurogenesis may be highly similar between hydrozoans and *Nematostella*. Additionally, i-cells give rise to cnidocytes, gland cells and, in the case of *Hydractinia,* to epithelial cell types. The pluripotency of a single i-cell has only recently been demonstrated in *Hydractinia* (Varley et al., 2022). *Hydra* i-cells, in contrast, are considered multipotent as they do not contribute to the two epithelial lineages (Hemmrich et al., 2012). Whether the potential of *Nematostella* Vasa2+/Piwi1+ stem-like cells comprises cnidocytes, gland cells and epithelial cells remains unknown. The level of potency of Vasa2+/Piwi1+

stem-like cells and how it compares to hydrozoan i-cells thus remains to be elucidated (see also section 1.2).

Hydrozoan i-cell populations have been proposed to be heterogeneous (Plickert et al., 2012), with *Hydra* presenting a germline-restricted subpopulation (Littlefield, 1985; Littlefield, 1991; Nishimiya-Fujisawa C. and Sugiyama, 1993). So far, I have found no evidence for the similar presence of a germline-restricted stem cell subpopulation within the Vasa2+/Piwi1+ stem-like cell population in *Nematostella* (see section 1.2).

In conclusion, I propose that the mesenterial Vasa2+/Piwi1+ stem-like cells of *Nematostella* presented in this work are likely homologous to hydrozoan i-cells. Further characterization of Vasa2+/Piwi1+ stem-like cells in *Nematostella*, together with studies in other non-hydrozoan cnidarians, will continue to reveal conserved and novel features of ASCs across cnidarians that will allow to reconstruct their evolutionary history.

3. The evolution of adult stem cells and body plasticity in animals

So far, populations of ASCs with germ/soma potential have been described in highly regenerative bilaterian (e.g. botrylloid ascidians, acoels and planarians) and non-bilaterian animals (e.g. sponges, hydrozoan cnidarians and, as shown here, in the anthozoan *Nematostella vectensis*), revealing a clear link between the presence of these cells and high body plasticity. Conversely, animals with low or absent body plasticity such as insects, nematodes and vertebrates lack ASCs with germ/soma potential (see introduction for more details). The distribution of adult PriSCs across the animal tree has led to propose two potential evolutionary scenarios: (1) The presence of ASCs with germ/soma potential is an ancestral trait of Metazoa that was lost in ecdysozoan and vertebrate lineages; (2) the embryonic segregation of germline and soma is ancestral to animals, and ASCs with germ/soma potential have been acquired independently in sponges, cnidarians, planarians, acoels and botrylloid ascidians. The high body plasticity of non-bilaterians and the presence of ASCs with germ/soma potential in sponges and hydrozoan cnidarians suggest that these may be common traits among non-bilaterian animals, which would support the first scenario. My findings indicate

that ASCs with germ/soma potential may be an ancestral trait to cnidarians, further supporting this assumption. In Bilateria, however, the presence of ASCs with germ/soma potential has only been demonstrated in planarians, botrylloid ascidians and acoels. While descriptions of elevated body plasticity in annelids or feather stars indicate that these animals may present ASCs with germ/soma potential, further evidence is needed. Recently, a study on diverse planarian species has shown that whole-body regeneration may not be ancestral to this phyla, suggesting the independent evolution of this trait along different planarian lineages (Vila-Farré et al., 2022). Altogether, the lack of data available on ASCs in a diversity of non-bilaterian and bilaterian phyla renders the question of ASC evolution currently difficult to resolve. Future studies addressing the presence and potential of ASCs in phyla such as ctenophores, staurozoans and scyphozoan cnidarians, and in diverse taxa within bilaterian phyla will allow a better understanding of ASC evolution.

4. Lessons from animal adult stem cells and potential applications

Independently of the evolutionary history of animal ASCs, the genetic toolkit underlying adult pluripotency and germ cell determination (i.e. GMP marker genes such as *vasa* and *piwi*) appears highly conserved across the animal tree. While in ecdysozoans and vertebrates, most of these genes are specifically expressed in the germline after embryogenesis, some have been shown to be expressed in somatic tissues in certain adult contexts. An example is cancer development, in which cells acquire the ability to indefinitely proliferate and become metastasizing by reactivating germline/multipotency genes such as *vasa* and *pl10* orthologs in a process known as gametic recapitulation (De Keuckelaere et al., 2018; Janic et al., 2010; Kerr et al., 2019; Poon et al., 2016; Pryszlak et al., 2021; Ross et al., 2014). The blastema of uniquely regenerative vertebrate animals such as the axolotl constitutes another example where genes such as *piwi* orthologs are upregulated and required for limb regeneration (Zhu et al., 2012). Remarkably, induced pluripotent stem cells also display an upregulation of *vasa* and *piwi* genes (Mikkelsen et al., 2008). Research of ASCs in organisms able to perform whole-body regeneration while avoiding cancer can thus help elucidating key molecular mechanisms regulating adult pluripotency, normally latent in

ecdysozoan and vertebrate lineages, and, eventually, open paths for the development of cancer treatments and stem cell-based regenerative therapies (Kukhanova et al., 2020; Lai and Aboobaker, 2018; Oviedo and Beane, 2009).

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