ISSN 1062-3604, Russian Journal of Developmental Biology, 2009, Vol. 40, No. 2, pp. 57–68. © Pleiades Publishing, Inc., 2009.
Original Russian Text © V.V. Isaeva, A.V. Akhmadieva, Ya.N. Aleksandrova, A.I. Shukalyuk, 2009, published in Ontogenez, 2009, Vol. 40, No. 2, pp. 83–96.

REVIEWS

Morphofunctional Organization of Reserve Stem Cells Providing for Asexual and Sexual Reproduction of Invertebrates

V. V. Isaeva[a,b], A. V. Akhmadieva[a], Ya. N. Aleksandrova[a], and A. I. Shukalyuk[a]

[a]*Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences, ul. Pal'chevskogo 17, Vladivostok, 690041 Russia*
[b]*Institute of Problems of Ecology and Evolution of the Russian Academy of Sciences, Leninskii pr. 33, Moscow, 119071 Russia*
e-mail: vv_isaeva@mail.ru

Received December 22, 2007; in final form, May 15, 2008

Abstract—Published and original data indicating evolutionary conservation of the morphofunctional organization of reserve stem cells providing for asexual and sexual reproduction of invertebrates are reviewed. Stem cells were studied in representatives of five animal types: archeocytes in sponge *Oscarella malakhovi* (Porifera), large interstitial cells in colonial hydroid *Obelia longissima* (Cnidaria), neoblasts in an asexual race of planarian *Girardia tigrina* (Platyhelmintes), stem cells in colonial rhizocephalans *Peltogasterella gracilis, Polyascus polygenea*, and *Thylacoplethus isaevae* (Arthropoda), and colonial ascidian *Botryllus tuberatus* (Chordata). Stem cells in animals of such diverse taxa feature the presence of germinal granules, are positive for proliferating cell nuclear antigen, demonstrate alkaline phosphatase activity (a marker of embryonic stem cells and primary germ cells in vertebrates), and rhizocephalan stem cells express the *vasa*-like gene (such genes are expressed in germline cells of different metazoans). The self-renewing pool of stem cells is the cellular basis of the reproductive strategy including sexual and asexual reproduction.

DOI: 10.1134/S1062360409020015

Key words: asexual reproduction, stem cells, germinal granules, *vasa* gene

Two major approaches to determine primary germ cells are recognized—preformation (asymmetric distribution of sexual determinants of maternal origin in early embryogenesis) observed in some multicellular animals, and epigenesis (induction by signals of somatic cell environment) observed in mouse with germline development after blastocyst implantation (Saffman and Lasko, 1999; Wylie, 1999; Houston and King, 2000; Extavour and Akam, 2003; Saitou et al., 2003; Hayashi et al., 2007; Travis, 2007), sea urchin (Juliano et al., 2006), some insects (Chang et al., 2006), and other organisms (Extavour and Akam, 2003; Travis, 2007). No early separation of germline cells is observed in colonial organisms (Buss, 1999): in animals with asexual reproduction, the line of reserve stem cells that can differentiate into germ and somatic cells is maintained throughout their life. Such capacity of stem cells (neoblasts) is particularly pronounced in planarians (Agata and Watanabe, 1999; Shibata et al., 1999; Peter et al., 2001; Isaeva et al., 2005). Stem cells of this kind differentiating into germ and somatic ones include archeocytes in sponges (Simpson, 1974; Müller, 2006), interstitial cells in cnidarians (Bode et al., 1996), and neoblasts in turbellarians (Gschwentner et al., 2001; Peter et al., 2001; Sato et al., 2001; Orii et al., 2005). Colonial ascidian cells capable to differentiate into both germ and somatic ones are referred to as stem cells (Stoner et al., 1999; Weissman, 2000; Laird et al., 2005). In line with this trend, we designated cells identified in budding stolons of the interna in representatives of colonial rhizocephalans as stem cells: they are capable of self-renewal and differentiation into somatic and germ cells of blastozooids (Isaeva et al., 2001, 2004, 2008; Shukalyuk et al., 2005, 2007; Isaeva and Shukalyuk, 2007).

In this review, ultrastructural and some cytochemical, immunochemical, and molecular properties of stem cells in vertebrates with asexual reproduction were considered. We studied the morphofunctional organization of stem cells in representatives of five types of multicellular animal: sponge *Oscarella malakhovi* (Porifera); colonial hydroid *Obelia longissima* (Cnidaria); asexual race of planarian *Girardia* (*Dugesia*) *tigrina* (Platyhelmintes); colonial rhizocephalans *Peltogasterella gracilis*, *Polyascus polygenea* and *Thylacoplethus isaevae* (Arthropoda); and colonial ascidian *Botryllus tuberatus* (Chordata). The germinal granules were studied as an ultrastructural marker and key organelle of germline and reserve stem cells in these invertebrates. The product of genes related to the evolutionary conserved *vasa* gene expressing in germline cells in variables metazoans was used as a molecular marker. Proliferating cell nuclear antigen (PCNA) was used as a marker of cell reproduction in the self-renewing lineage of stem cells. The assay

for alkaline phosphatase activity was used as a cytochemical marker of stem cells as it was used elsewhere to identify the primary germline and embryonic stem cells in mammals. On the basis of the published and original data, we propose the common structural and functional organization of reserve stem cells in animals with asexual reproduction and germline cells.

TERMINOLOGY

The statement that there is no generally accepted universal definition of stem cells opens the *Stem Cell Biology* handbook (2001, p. 1). Stem cells are considered capable of self-renewal and differentiation into specialized cells; depending on the cytodifferentiation potential, totipotent, pluripotent, multipotent, oligopotent, and unipotent stem cells are recognized (Weissman, 2000; *Glossary…; Stem Cell Information*). The current terminology was largely developed in studies of mammalian stem cells, but has not been unified even for them. For instance, totipotent cells are considered as cells capable of differentiation into all cell types in mammalian body including those of extraembryonic tissues (*Glossary…*) or as cells capable of forming the entire body so that only zygote and early embryonic cells are totipotent (*Stem Cell Information*; Smith, 2001, 2006). At the same time, the term totipotency is used in biology to mark the potential of a cell or its progeny to produce all cell types (*Developmental…*; Gilbert, 2006; Seydoux and Braun, 2006). Some researchers believe that the totipotent potential is maintained by female germline cells (Weissman, 2000; Seydoux and Braun, 2006; Strome and Lehman, 2007); while others consider germline cells as pluripotent only (Hogan, 2001; Smith, 2006). The definition of pluripotent stem cells is even more conflicting: either cells capable of giving rise to many but not all cell types of the organism (*Developmental…*; Winslow, 2001), to cells of all three germ layers (*Stem Cell Information*), to all cell types of the implanted embryo and developing organism except trophoblastic and placental cells (*Glossary…*), or to all embryonic cell types including extraembryonic ones (Smith, 2006). Multipotent cells can give rise to many cell types; oligopotent, to few cell types; unipotent, to a single type of differentiated cells (*Glossary…*).

Cultured mammalian embryonic stem cells that can differentiate into germline cells and to all somatic cell types under specific conditions are commonly considered as pluripotent (Smith, 2001, 2006; *Glossary…*), although they are sometimes considered as totipotent (Reddy et al., 1992; Pesce et al., 1999). We adhere to the totipotency definition accepted in developmental biology and to the concept of totipotency maintenance by female germline cells (see above). In our view, the ability of embryonic stem cells to differentiate into female germ cells (Hübner et al., 2003; Daley, 2007; Kerkis et al., 2007) indicates their totipotency.

Stem cells of invertebrates with asexual reproduction capable of differentiation into both germline and all somatic cell types are traditionally referred to as totipotent; while stem cells giving rise to germline and many but not all somatic cell types of the body are sometimes referred to as multipotent. For instance, archeocytes in sponges and neoblasts in turbellarians are considered totipotent, while large interstitial cells in cnidarians are considered multipotent (see below). The considered stem cells of invertebrates providing for sexual and asexual reproduction are referred to as reserve stem cells here. We believe that the capacity of such cells to differentiate into female germline cells allow them to be considered as totipotent irrespective of the range of their somatic derivatives.

To avoid confusion, note that we have excluded cambial stem cells from consideration; such cells provide for the physiological and reparative renewal of tissues and have limited morphogenetic potential (multi-, oligo-, and unipotency), but are incapable of extensive migration; asymmetric mitosis is considered a priority for cambial stem cells by some (Wolpert, 1988).

CAPACITY OF RESERVE STEM CELLS IN INVERTEBRATES TO DIFFERENTIATE INTO GERMLINE AND SOMATIC CELLS

Archeocytes in sponges can differentiate into gametogenic and somatic cells (Simpson, 1984; Müller, 2006) and are considered as totipotent cells (Müller, 2006). Nikitin (1974) has experimentally confirmed that uniform cell aggregates formed from isolated nucleolar amoebocytes (archeocytes) in freshwater sponge *Ephydatia fluviatilis* L. can develop into a sponge body.

Large interstitial cells in hydra and other cnidarians can produce both germline cells and some but not all somatic cell types, since epidermal and gastrodermal cells are capable of mitotic reproduction (Campbell, 1974; Thomas and Edwards, 1991; Bode, 1996) and, thus, were assigned to multipotent cells (Mochizuki et al., 2001). According to our data, gonial cells and early oocytes in hydroid *O. longissima* can be distinguished from large interstitial cells only by a larger size (Akhmadieva et al., 2005; Akhmadieva, 2008).

Neoblasts in asexually reproducing planarians and other turbellarians can differentiate into both germ line and somatic cells of all types, and are commonly considered as totipotent cells (Agata and Watanabe, 1999; Shibata et al., 1999; Peter et al., 2001; Orii et al., 2005). Neoblasts in an asexual race of planarian *G. tigrina* can become gonial cells: one of asexual individuals that reproduced exclusively by architomy has laid cocoons after spontaneous sexualization; histological and ultrastructural study of this planarian demonstrated the presence of gonads, gonial cells, and oocytes (Isaeva et al., 2005).

In the interna of colonial rhizocephalans *P. polygenea* and *P. gracilis* stem cells give rise to all early primordia in blastozooids; then they differentiate into all somatic cell types of the body developing through blastogenesis and migrate to the developing ovary to become oogonia (Isaeva et al., 2004; Shukalyuk et al., 2005; Isaeva and Shukalyuk, 2007). The potential of stem cells in the colonial interna of rhizocephalans is similar to that of neoblasts in planarians, and we consider such cells as totipotent.

Fusion of the vascular systems of colonies in ascidian *Botryllus schlosseri* can lead to the replacement of sexual and somatic cells in one colony with those from another one (Pancer et al., 1995; Stoner and Weissman, 1996; Stoner et al., 1999; Weissman, 2000; Laird and de Tomaso, 2004/2005). In colonial ascidians, germline and somatic cells differentiate from circulating blood cells (Pancer et al., 1995; Stoner and Weissman, 1996; Stoner et al., 1999). It remains unclear if the germline and somatic lineages separate or they descend from the same initial stem cell population in botryllids (Stoner et al., 1999; Broun and Swalla, 2007); accordingly, stem cells in ascidians are considered as pluripotent (Sunanaga et al., 2006) or totipotent (Stoner et al., 1999; Laird and Weissman, 2004). Recently, the differentiation of *Botryllus primigenus* hemoblasts into female germline cells, and the primary germline cells are morphologically indistinguishable from hemoblasts (Sunanaga et al., 2006).

SELF-RENEWAL OF STEM CELLS IN INVERTEBRATES

Stem, primary germ, and gonial cells have a common property—self-renewal through mitotic reproduction over long periods or throughout life span of the organism (Houston and King, 2000; *Stem Cell Biology*, 2001; Hogan, 2000; Winslow, 2001). For instance, large interstitial cells in hydra and other cnidarians are stem cells continuously undergoing the mitotic cycle; the stem cell system in hydroids also includes epidermal and gastrodermal cells capable of mitotic reproduction (Campbell, 1974; Thomas and Edwards, 1991; Bode, 1996). Bromodeoxyuridine, a thymidine analogue incorporating into synthesized DNA, was used to reveal the proliferation of interstitial cells in hydra (Teragawa and Bode, 1990), neoblasts in flatworms (Gschwentner et al., 2001; Peter et al., 2001), and stem cells in annelids (Kozin et al., 2007). PCNA assay is used to reveal proliferation in vertebrate cells (Hall and Woods, 1990; Mueller and Wullimann, 2003). Such assay was also used to identify neoblasts in planarian *Girardia japonica* (Orii et al., 2005).

We used an immunochemical test to identify proliferating cells in colonial rhizocephalan *P. gracilis* and colonial hydroid *O. longissima* using antibodies against mouse PCNA. Stem cells in budding stolons proved to be the only positive cell type in the *P. gracilis* interna (Shukalyuk et al., 2005; Isaeva and Shukalyuk, 2007). Such test in *O. longissima* has revealed a more intense staining of large interstitial and gonial cells, although reproducing epidermal and gastrodermal cells were also positive (Akhmadieva et al., 2005; Akhmadieva, 2008).

High activity of telomerase, which protects stem cell chromosomes from replicative shortening, has been revealed in cells of sponge gemmules (Müller, 2002) as well as in cells of the embryos, gonads, and early buds in colonial ascidian *B. schlosseri* (Laird and Weissman, 2004).

GENERAL MORPHOLOGY AND MOTILITY OF STEM AND GONIAL CELLS IN INVERTEBRATES

The morphological structure of reserve stem cells in the studied representatives of Porifera, Cnidaria, Platyhelminthes, Arthropoda, and Chordata shares common properties: the absence of morphological signs of cytodifferentiation; high nuclear/cytoplasmic ratio; large nucleus with dispersed chromatin; large nucleolus and compact basophilic cytoplasm containing numerous free ribosomes, mitochondria, and perinuclear germinal granules. A similar morphology is typical of gonial cells in the studied invertebrates.

Asexual reproduction of sponges includes fragmentation, budding, and gemmule formation (Ivanova-Kazas, 1977; Simpson, 1984); archeocytes are the dominant cell type in the buds (Müller, 2006). Budding was also demonstrated in sponge *O. malakhovi* (Ereskovsky, 2006). According to our data, migrating archeocytes morphologically similar to those described previously in other sponge species actively participate in *O. malakhovi* budding (Akhmadieva, 2008).

In hydra and other cnidarians, large interstitial cells can actively migrate (Campbell, 1974; Thomas and Edwards, 1991; Bode, 1996). Interstitial and gonial cells in colonial hydroid *Obelia longissima* demonstrate the ultrastructural morphology typical for cnidarian stem and germline cells; numerous large interstitial cells migrate to the sites of bud formation in *O. longissima* (Akhmadieva et al., 2005; Akhmadieva, 2008).

Neoblasts and gonial cells in planarian *G. tigrina* (Isaeva et al., 2005) demonstrate the morphological structure similar to that described previously in other studies; turbellarian neoblasts can migrate to the injured surface and sites of gonad formation (Rieger et al., 1991; Auladell et al., 1993; Agata and Watanabe, 1999; Shibata et al., 1999).

The colonial organization in some rhizocephalans (Crustacea: Cirripedia: Rhizocephala) appears at the parasitic stage of their life cycle as a result of asexual reproduction (Høeg and Lützen, 1995; Kasyanov et al., 1997a, 1997b; Takahashi and Lützen, 1998; Isaeva et al., 1999, 2008; Isaeva and Shukalyuk, 2007); however, blastogenesis was unambiguously demonstrated only in some rhizocephalans species. We have found stem cells at the endoparasitic stage of the life cycle in colonial rhizocephalans *P. polygenea, P. gracilis* and *Th. isaevae* (Isaeva et al., 2001, 2003, 2004, 2008;

Rybakov and Shukalyuk, 2004; Shukalyuk et al., 2005, 2007; Isaeva and Shukalyuk, 2007). Undifferentiated stem cells have been found inside each early stolon bud; similar cells migrate within the stolon. Stem cells in rhizocephalans demonstrate all morphological properties shared by stem and gonial cells in other studied animals with asexual reproduction.

Hemoblasts of colonial ascidian *Botrylloides leachi* as well as of other ascidians are considered as putative totipotent or pluripotent stem cells (Burighel and Cloney, 1997; Cima et al., 2001; Sunanaga et al., 2006). During fusion of histocompatible colonies of ascidian *B. schlosseri*: the replacement of germline and somatic cells of one colony with those from the other one has been shown, and it was mediated by stem cells circulating in the vascular system (Pancer et al., 1995; Stoner et al., 1999; Wiseman, 2000; Laird and de Tomaso, 2004/2005; Laird et al., 2005), although stem cells have not been morphologically described in *Botryllus* ascidians. We have found stem cells in colonial ascidian *B. tuberatus*: undifferentiated stem cells capable of migration were identified in the early buds and vascular system of the colony (Akhmadieva et al., 2007). Stem cells in *B. tuberatus* are morphologically similar to hemoblasts in *B. leachi* (Cima et al., 2001) and other studied ascidians (Burighel and Cloney, 1997).

The morphofunctional organization of the primary germline and gonial cells in all studied representatives of multicellular animals share the same properties with reserve stem cells in sexually-reproducing invertebrates: high nuclear/cytoplasmic ratio, large nucleus with diffuse chromatin and large nucleolus, and basophilic cytoplasm containing structured germinal granules. The primary germline cells are known to emerge outside of the future gonad and later migrate into it using both amoeboid motility and passive morphogenetic movements (Wylie, 1999; Matova and Cooley, 2001; Kunwar and Lehmann, 2003; Travis, 2007).

GERMINAL GRANULES IN GERMLINE CELLS

It is common knowledge that differentiation of the primary germline and later gonial cells is associated with the functioning of the specialized cytoplasmic region called the germ (embryonic) plasm. The concept of preformed germ plasm (Keimplasma) with determinants of germline cells transmitted from generation to generation and providing for the germline continuity and inheritance of a sense of species-specific characters has been developed by Weissmann (1892, 1893).

The germ plasm contains the germinal granules (germ determinants) as RNA-rich granular or fibrillar material (not surrounded with a membrane), which can be used as an ultrastructural marker to identify germline cells and to follow their fate in ontogeny. The main germinal granules components include proteins, mRNAs, and noncoding RNAs (Seydoux and Braun, 2006). The germinal granules considered as specific organelles of germline cells (Saffman and Lasko, 1999; Amikura et al., 2001; Seydoux and Braun, 2006; Lim and Kai, 2007; Strome and Lehman, 2007) are also referred to as polar granules; germinal, chromatoid, perinuclear, or dense bodies; nuage (cloud in French); intermitochondrial cement, oosomes, etc. in different animals (Mahowald, 1971, 2001; Beams and Kessel, 1974; Eddy, 1975; Aizenshtadt, 1984; Ikenishi, 1998; Saffman and Lasko, 1999; Wylie, 1999; Houston and King, 2000; Isaeva and Reunov, 2001; Matova and Cooley, 2001; Chang et al., 2006; Seydoux and Braun, 2006; Strome and Lehnam, 2007). The presence of germinal (perinuclear) granules is an evolutionary conserved character of germline cells in multicellular animals. These specific organelles have been found in more than 80 species of seven animal types (Eddy, 1975). The structure of these organelles is similar, but they can be represented in cells of different organisms and at different life cycle stages as either not numerous large granules (bodies) or as a cloud (nuage) of fine-dispersed material. In oogenesis, the germinal bodies transform morphologically but do not disappear in female germ cells throughout the life cycle: for instance, the polar granules are gradually replaced with nuage during polar cell migration in *Drosophila.* The continuity of the maternally inherited germ plasm material throughout the life cycle has been demonstrated in *Drosophila, Xenopus*, and nematode (Mahowald, 1971, 2001; Ikenishi, 1998).

Proteins localized in the germ plasm granules are involved in the germline cell determination, and their genes are evolutionary conserved in different studied metazoans from sponges to mammals (Ding and Lipshitz, 1993a, 1993b; Ikenishi, 1998; Saffman and Lasko, 1999; Houston and King, 2000; Matova and Cooley, 2001; Mochizuki et al., 2001; Chang et al., 2006; Seydoux, Braun, 2006; Klenov et al., 2007; Strome and Lehman, 2007).

As far as is known, the germinal granules rich in RNA and RNA-binding proteins are involved in mRNA localization, protection, and translation control (Saffman and Lasko, 1999; Extavour and Akam, 2003; Leatherman and Jongens, 2003; Saitou et al., 2003; Seydoux and Braun, 2006; Hayashi et al., 2007; Lim and Kai, 2007; Strome and Lehman, 2007). The germinal granules are thought to function as a specific regulatory center preventing the expression of somatic differentiation genes, maintaining the genomic totipotency in germline cells (Seydoux and Braun, 2006), and protecting them from somatic fate (Strome and Lehman, 2007).

Recently, processing bodies (P-bodies) have been found in somatic cells; their function is translation and they are considered as a structural and functional analog of the germinal (perinuclear) granules (Seydoux and Braun, 2006; Kotaja et al., 2006; Klenov et al., 2007).

GERMINAL GRANULES IN STEM CELLS OF INVERTEBRATES WITH ASEXUAL REPRODUCTION

The germinal granules in stem cells of invertebrates, whose life cycle includes asexual reproduction, were previously known only in hydra and planarians. The electron-dense bodies similar or identical to the germinal granules in germline cells were found in large interstitial cells of hydra *Pelmatohydra robusta* (Noda and Kanai, 1977; Mochizuki et al., 2001) and in neoblasts of planarians (Hori, 1982; Rieger et al., 1991; Auladell et al., 1993; Agata and Watanabe, 1999; Shibata et al., 1999). The number and size of the germinal granules (dense bodies) in *P. robusta* decreases as somatic cells (cnidoblasts) differentiate from interstitial cells and vice versa increases during early oogenesis (Noda and Kanai, 1977). Similarly, the germinal (chromatoid) bodies in planarian neoblasts disappear in the course of their differentiation into somatic cells, which is considered a marker of their function related to totipotency maintenance (Shibata et al., 1999).

In the cytoplasm of archeocytes in sponge *O. malakhovi* we have found small germinal granules with a typical morphology located near the nuclear envelope (Akhmadieva, 2008). The electron-dense germinal bodies that we have found in large interstitial cells and oogonia of hydroid *O. longissima* have similar ultrastructure to the dense bodies in germline and interstitial cells of *P. robusta* (Noda and kanai, 1977) as well as in oocytes of *Hydra carnea* (Honegger et al., 1989) and other cnidarians (Thomas and Edwards, 1991). Such germinal granules proved common for large interstitial cells as well as for gonial cells and oocytes of *O. longissima* (Akhmadieva et al., 2005; Akhmadieva, 2008). The germinal granules (chromatoid bodies) have been found near the nuclear envelope (often in contact with nuclear pores) surrounded by mitochondria in neoblasts and gonial cells of planarian *G. tigrina* (Isaeva et al., 2005).

The cytoplasm of embryonic and reserve stem cells in the studied rhizocephalans species proved to contain germinal granules morphologically similar to those in germline cells of *Drosophila* and other multicellular organisms. In particular, stem cells in rhizocephalan *P. gracilis* feature the presence of the germinal bodies with a typical ultrastructural morphology; all or most blastomeres contain large germinal granules in cleaving *P. gracilis* embryos (Shukalyuk et al., 2005, 2007; Isaeva and Shukalyuk, 2007).

Hemoblasts in colonial ascidian *B. primigenus* contain neither electron-dense material nor mitochondrial clusters; such organelles appear later in differentiating oogonia and oocytes (Sunanaga et al., 2006). In the cytoplasm of some stem cells in the early buds of ascidian *B. tuberatus* we have found small electron-dense bodies in dispersed nuage, which is not uncommon in vertebrates, rather than with large germinal granules in other invertebrates (Akhmadieva et al., 2008).

Thus, reserve stem cells in all studied asexually reproducing sponges, cnidarians, turbellarians, arthropods, and chordates feature the presence of the germinal granules.

EXPRESSION OF *VASA* AND RELATED GENES IN GERMLINE AND STEM CELLS

The protein products of the *Drosophila vasa* gene and its homologs, RNA helicases of the protein family containing conserved DEAD box sequences, are a component of the germinal granules and a universal marker of germline cells in metazoans (Saffman and Lasko, 1999; Raz, 2000; Extavour and Akam, 2003; Chang et al., 2006; Juliano et al., 2006). DEAD family proteins are involved in splicing, nucleocytoplasmic transport, translation initiation, and RNA degradation in all eukaryotes (Raz, 2000). Vasa protein is required for the formation and maintenance of the structural organization of the polar (germinal) granules and, presumably, for the totipotency maintenance in germline cells (Houston and King, 2000; Raz, 2000; Seydoux and Braun, 2006; Strome and Lehman, 2007). In animals with solely sexual reproduction, *vasa* expression is limited to germline cells throughout their development—from early embryogenesis to gametogenesis (Raz, 2000; Mochizukie, 2001; Sunanaga et al., 2006). Selective expression of a *vasa*-like gene was detected in germline cells of cnidarian *Nematostella vectensis* (Extavour et al., 2005) and crustacean (amphipod) *Parhyale hawaiensis* (Extavour, 2005), in the primary germline cells and their precursor blastomeres of ascidian *Ciona intestinalis* (Fujimura and Takamura, 2000; Takamura et al., 2002), and in human oogenic cells (Castrillon et al., 2000; Stoop et al., 2005). The genes structurally similar to *vasa* have been found in all studied multicellular animals (from sponges and cnidarians to vertebrates); the products of these genes (RNA and/or protein) is localized to the germinal granules in germline cells of nematodes, insects, turbellarians, chaetognaths, and vertebrates. Vasa protein is considered as a key determinant of the fate of these cells (Hay et al., 1988; Ding and Lipshitz, 1993a, 1993b; Ikenishi, 1998; Saffman and Lasko, 1999; Shibata et al., 1999; Castrillon et al., 2000; Houston and King, 2000; Matova and Cooley, 2001; Mochizuki et al., 2001; Carre et al., 2002; Seydoux and Braun, 2006). Antibodies against Vasa protein react with both polar granules and nuage in *Drosophila* (Hay et al., 1988), which confirms the functional identity of these structures (Mahowald, 2001). The presence of Vasa-like proteins in the germ plasm of different animals indicates the conservation of similar and molecular mechanisms underlying the formation and maintenance of the germ plasm (Saffman and Lasko, 1999; Raz and 2000; Houston and King, 2000; Carre et al., 2002; Extavour and Akam, 2003; Juliano et al., 2006).

In polyembryonic insect *Copidosoma floridanum*, the secondary embryos develop either into normal lar-

vae and then into fertile wasps or into soldier larvae whose function is defense. At the stage of four blastomeres, one of them is Vasa-positive, and it gives rise to primary germline cells; precisely these embryos become fertile imagoes. The caste of soldiers with no germline cells develops from the embryos expressing no *vasa* homolog or after experimental eliminating the Vasa-positive blastomere, which confirms the involvement of this protein in germline and caste determination in *C. floridanum* (Donnell et al., 2004; Corley et al., 2005).

The presence of a Vasa-like protein was demonstrated not only in germline cells but also in large interstitial cells of hydra *P. robusta* (Mochizuki et al., 2001) and neoblasts of planarians (Shibata et al., 1999); hence, the product of *vasa*-related genes can be a useful molecular marker of stem cells in invertebrates with asexual reproduction (Shibata et al., 1999; Mochizuki et al., 2001). *Vasa*-like gene expression was demonstrated in oogonia, oocytes, and primary germline cells morphologically indistinguishable from hemoblasts in ascidian *B. primigenus* (Sunanaga et al., 2006). Expression of *vasa*-like genes has been revealed in stem cells of annelids (Kozin et al., 2007).

We have found evolutionary conserved genes of the DEAD family in rhizocephalans *P. polygenea* and *Clistosaccus paguri* in particular, *vasa*-like genes closely related in structure to such genes in other arthropods. The localization of the *vasa*-like gene was limited to gametogenic and stem cells as well as to the germinal granules in all or most blastomeres in the early *P. polygenea* embryos (Shukalyuk et al., 2007; Isaeva and Shukalyuk, 2007).

Thus, the product of *vasa*-related genes has been found in both germline and reserve stem cells of planarians, hydra, and colonial rhizocephalan *P. polygenea.* In planarians (Shibata et al., 1999) and rhizocephalan *P. polygenea* (Shukalyuk et al., 2007), the product of these genes was selectively localized to the germinal granules. Thus, the maintenance of the stem cell morphofunctional organization involves evolutionary conserved developmental mechanisms common for asexually reproducing multicellular animals.

ROLE OF MITOCHONDRIA IN GERMINAL GRANULE BIOGENESIS

The contact with mitochondria is a typical property of structured germinal granules in diverse multicellular animals (Aizenshtadt, 1984; Isaeva and Reunov, 2001; Matova and Cooley, 2001; Carré et al., 2002). In *Drosophila*, planarians, and *Xenopus*, ribosomal RNA of mitochondrial origin and some other products of the mitochondrial genome have been revealed in the embryonic germinal granules (Ding and Lipshitz, 1993a, 1993b; Kobayashi et al., 1993, 1998, 2005; Kashikawa et al., 1999). The germinal granules are commonly surrounded with polysomes (Mahowald, 2001); polysomes in *Drosophila* embryos were shown to contain ribosomes similar to mitochondrial ones by size properties (Amikura et al., 2001; Kobayashi et al., 2005). Mitochondrial rRNA can restore polar cell formation in *Drosophila* embryos after UV irradiation (Kobayashi and Okada, 1989), while the absence of extramitochondrial 16S rRNA (synthesized in mitochondria) suppresses germline cell formation (Iida and Kobayashi, 1998). In *Xenopus* 16S rRNA can be found outside of mitochondria only in the germ plasm granules (Kobayashi et al., 1998). The products of both nuclear and mitochondrial genomes are thought to be critical for the structural organization and functioning of germ plasm determinants (Kobayashi et al., 1993, 1998, 2005; Ding and Lipshitz, 1993, 1993b; Iida and Kobayashi, 1998; Kashikawa et al., 1999).

Mitochondrial (both large and small) ribosomal RNA has also been detected in the chromatoid bodies in turbellarian neoblasts (Sato et al., 2001). The presence of mitochondrial rRNA outside of mitochondria in association with the germinal granules has been generally accepted; it becomes apparent that mitochondrial rRNA and other products of the mitochondrial genome are involved in the formation of germline cells in diverse multicellular animals (Ikenishi, 1998; Saffman and Lasko, 1999; Houston and King, 2000; Kloc et al., 2000; Mahowald, 2001; Amikura et al., 2001; Matova and Cooley, 2001; Leatherman and Jongens, 2003; Seydoux and Braun, 2006). Moreover, mitochondrial 16S rRNA has been found in the nuclei of spermatogonia, spermatocytes, and spermatids of mammals (Villegas et al., 2002). The transport of the large and small rRNA subunits from mitochondria to the germinal granules is no longer questioned, but the underlying mechanism is considered unprecedented and enigmatic (Ding and Lipshitz, 1993a, 1993b; Amikura et al., 2001).

Our ultrastructural study of mitochondria in the germ plasm of gonial and stem cells exposes this transport mechanism of mitochondrial products to the germinal granules. Degradation of the mitochondrial outer membrane, release of mitochondrial matrix material to the cytoplasm, and its transformation into the germinal granules was observed in gonial cells of sea urchin (Reunov et al., 2000), trepang, and flatfish (Reunov et al., 2004). The ultrastructural evidence of mitochondrial origin of the germinal granules (chromatoid bodies) in gonial cells and neoblasts of planarian *G. tigrina* have been obtained—transformation of mitochondrial matrix with inner membrane cristae into the germinal bodies (Isaeva et al., 2005). The mitochondrial derivatives deprived of the outer membrane but still containing inner membrane cristae is a usual picture observed in the germ plasm of stem and gonial cells in the studied representatives of diverse taxa including sponge *O. malakhovi* and hydroid *O. longissima* (Akhmadieva, 2008). The release of the mitochondrial matrix material in the germ plasm is the way to incorporate the mitochondrial derivatives into the germinal granules, which mediates the involvement of mitochondrial genome products in the biogenesis of the macromolecular complex of

germinal determinants (Isaeva and Reunov, 2001; Isaeva et al., 2005). We assume that molecular components of the germinal granules are transported to the nucleus, which programs the totipotency maintenance, and molecular information flows connecting the nucleus, mitochondria, and germinal granules (Isaeva et al., 2005).

The export of mitochondrial rRNA from mitochondria to the polar granules in *Drosophila* depends on the activity of nuclear genes *oskar, vasa*, and *tudor* (Saffman and Lasko, 1999; Matova and Kooley, 2001; Amikura et al., 2001). Vasa protein or its homolog, a component of the germinal granules in different animals, has been found in the mitochondrial matrix of germline cells in *Xenopus* embryos (Watanabe et al., 1992). Similarly, the protein encoded by the nuclear gene *tudor* is present both in the polar granules and inside mitochondria in the early *Drosophila* embryos (Ding and Lipshitz, 1993). In addition to these proteins, the germ determinants include many more components encoded in the nuclear genome (Ikenishi, 1998; Saffman and Lasko, 1999; Houston and King, 2000; Matova and Cooley, 2001). The formation of the germinal (perinuclear, chromatoid) bodies is a complex assembly process involving RNA-binding protein complexes specifically interacting with each other and different RNA species (Ding and Lipshitz, 1993, 1993b; Williamson and Lehman, 1996; Ikenishi, 1998; Matova and Cooley, 2001; Klenov et al., 2007; Strome and Lehman, 2007).

The maintenance of the preexisted structural and functional organization of the germ plasm determinants is likely governed by ancient conserved mechanisms common for all multicellular animals. We believe that the specific complex of proteins and RNAs in the germinal granules is the regulatory center of the macromolecular organization including the maternal inheritance structures (Isaeva and Reunov, 2001; Isaeva et al., 2005).

ALKALINE PHOSPHATASE ACTIVITY

Histochemically detectable high level of alkaline phosphatase activity has become an empirical marker of mammalian primary germline and embryonic stem cells in vivo and in vitro (Chiquoine, 1954; Mintz, 1959; Merchant-Larios et al., 1985; Talbot et al., 1993; Lacham-Kaplan, 2004). High alkaline phosphatase activity was also found in cultures of embryonic stem cells of birds (Pain et al., 1996) and fishes (Hong et al., 1998). Alkaline phosphatase was also immunochemically detected in human oogonial cells (Stoop et al., 2005).

High alkaline phosphatase activity is observed only in the cytoplasm of stem cells in the studied colonial rhizocephalans; all other cells and tissues of the endoparasitic interna have no such activity (Isaeva et al., 2003; Shukalyuk et al., 2005; Isaeva and Shukalyuk, 2007). In blastomeres of cleaving *P. polygenea* embryos, high alkaline phosphatase activity is locally detected in the germinal granules (Shukalyuk et al., 2005; Isaeva and Shukalyuk, 2007). Histochemical assay for alkaline phosphatase reveals intense staining of interstitial and gonial cells in hydroid *O. longissima* (Akhmadieva et al., 2005; Akhmadieva, 2008) as well as of the early buds and a fraction of hemocytes (apparently, stem cells hemoblasts) in ascidian *B. tuberatus* (Akhmadieva et al., 2007).

Specific brick-red staining of reserve stem cells in the studied colonial cnidarians, arthropods, and chordates is similar in color and intensity to that of cultured mouse embryonic stem cells used for reference.

REPRODUCTIVE STRATEGY INCLUDING ASEXUAL REPRODUCTION

Reproductive strategy within the wide frames of ecology and evolution is considered as a complex of adaptive characters and properties of reproductive and developmental biology from subcellular and cellular to specific and biocenotic levels (Kasyanov, 1989, 2001). Blastogenesis and colonial structure of representatives of rhizocephalans involve the radical and evolutionary secondary rearrangement of the initial reproductive strategy of crustaceans due to their transition to parasitic life. Asexual reproduction and the more so coloniality are quite untypical for arthropods: polyembryony is observed in a few insects in association with either parasitism or viviparity (Ivanova-Kazas, 1977, 1981). The metamorphosis of larvae typical of Cirripedia leads to the loss of an individual organism and its systems of organs as well as of segmentation in female larvae. The metamorphoses of male larvae leads to even further reduction—a population of spermatogenic cells cultured in the female body (stated differently, parasitic). As a result, rhizocephalans demonstrate gonochorism with the extreme sexual dimorphism with dwarf males reduced to germline cells; no such reduction is observed in other multicellular animals (Kasyanov et al., 1997a, 1997b, 1998, 1999; Kasyanov, 2001).

The presence of germinal bodies (with the selective localization of *vasa*-related transcript) in all or most blastomeres in cleaving embryos of rhizocephalans *P. polygenea* indicates a radical rearrangement of the initial arthropod type of early development typical of the whole Ecdysozoa branch—and an evolutionary jump from determined mosaic cleavage with the early separation of the germ cell line (preformation) to regulative development with epigenesis.

Following the fate of the germ layers after metamorphosis in rhizocephalans is impracticable (Ivanova-Kazas, 1979; Kasyanov et al., 1998). The attempts to correlate the early bud components in blastogenesis of other colonial animals with the germ layers in embryogenesis are also not very successful (Ivanova-Kazas, 1977). We believe that totipotent stem cells play the key role in blastogenesis in invertebrates with asexual reproduction. The finding of undifferentiated stem cells in the early buds of ascidian *B. tuberatus* (Akhmadieva

et al., 2007; Akhmadieva, 2008) addresses the problems of the previously proposed development of all three germ layers from a single epithelial layer, atrial epithelium, during parallel budding of botryllids (Berrill, 1961; Ivanova-Kazas, 1978). Colonial rhizocephalans and *Botryllus* ascidians fit to the group of multicellular animals whose reproductive strategy includes asexual reproduction realized through reserve stem cells.

Let us sum up. Stem cells in asexually reproducing animals from various taxa such as sponges, cnidarians, flatworms, arthropods, and chordates share evolutionary conserved properties presumably related to the totipotency maintenance. Stem cells in the studied invertebrates with asexual reproduction can differentiate into gametes and are similar to germline cells. They have a similar morphology including the ultrastructural level (the presence of the germinal bodies; express a *vasa*-related gene; have alkaline phosphatase activity; and are capable of mitotic reproduction. The primary germ line and reserve stem cells in colonial invertebrates are capable of extensive migrations in the body targeted to the gonads, asexual reproduction sites, or, in solitary species with asexual reproduction (turbellarians), to the wound surface resulting from fission or damage.

Primary germ line and totipotent stem cells share many morphological characters and rely on the activity of related genes; their evolutionary and ontogenetic relationship is proposed (Weissman, 2000; Extavour and Akam, 2003; Hayashi et al., 2007; Travis, 2007). The common properties of these cell lineages reflect their common origin from totipotent cells in the early embryo (Weissman, 2000). Note also the common properties of the morphofunctional organization of germ line and totipotent stem cells in multicellular animals with asexual reproduction (Shukalyuk and Isaeva, 2005a, 2005b; Isaeva et al., 2007). Both germline and reserve stem cells in animals with asexual reproduction derive in the early embryogenesis from totipotent blastomeres or their yet totipotent derivatives. We believe that evolutionary and ontogenetically related cells in the early embryos, primary germ line and totipotent stem cells, belong to the pool of reserve cells that can realize the whole developmental program. The concept of set-aside cells retaining a wide morphogenetic potential, which is realized after metamorphoses, was elaborated by Davidson and other scientists (Davidson, 1991; Davidson et al., 1995; Peterson et al., 1997; Jenner, 2000; Collins and Valentine, 2001) for animals with indirect larval development. During asexual reproduction, reserve stem cells in invertebrates retain the capacity of differentiation into both somatic and germline cells, i.e., unlimited morphogenetic potential or totipotency. Such cells in invertebrates with asexual reproduction potentially able to realize the entire program of embryonic development and blastogenesis are similar in their potential to mammalian embryonic stem cells, although the latter are artificial cell systems cultured in vitro. In parasitic rhizocephalans, stem cells are "cultured" in the host hemolymph or, in free-living invertebrates, in their own body. Germline and totipotent stem cells are privileged "predatory" cells that can survive starvation through "cannibalism" (Kerszberg and Wolpert, 1998) and are disposed to "parasitism" (Pancer et al., 1995; Laird and de Tomaso, 2004/2005).

Thus, published and original data indicate evolutionary conservation and similarity of the studied morphofunctional properties of reserve stem cells in metazoans with asexual reproduction (from sponges and cnidarians to chordates) and germline cells. In invertebrates with asexual reproduction, stem cells can differentiate into both germline and somatic cells; thus, they represent a source of cells for the life strategy realization including sexual and asexual reproduction.

ACKNOWLEDGMENTS

Our work would be impossible without great, invaluable, and direct contribution and support of Vladimir L. Kasyanov, Academician of the Russian Academy of Sciences, Director of the Institute of Marine Biology of the Far Eastern Branch of the Russian Academy of Sciences, scientific leader of the Laboratory of Embryology founded by him at the Institute, and head of a leading scientific school, tragically died on October 1, 2005. This work was supported by the Russian Foundation for Basic Research (project nos. 06-04-48744, 06-04-63092, and 06-04-96039) and Far Eastern Branch of the Russian Academy of Sciences (project no. 06-III-A-06-162).

REFERENCES

Agata, K. and Watanabe, K., Molecular and Cellular Aspects of Planarian Regeneration, *Cell Devel. Biol.*, 1999, vol. 10, pp. 377–383.

Aizenshtadt, T.B., *Tsitologiya oogeneza* (Cytology of Oogenesis), Moscow: Nauka, 1984.

Akhmadieva, A.V., Morphofunctional Study of Stem Cells in Invertebrates with Reproductive Strategy Including a Sexual Reproduction, *Extended Abstract of Cand. Sci. (Biol.) Dissertation*, Vladivostok: Institut biologii morya, 2008.

Akhmadieva, A.V., Shulalyuk, A.I., and Isaeva, V.V., Interstitial Cells in Reproductive Strategy of Colonial Hydroid *Obelia longissima, Mol. Biol. Cell.*, 2005, vol. 16, no. 11 suppl., p. 752a.

Akhmadieva, A.V., Shukalyuk, A.I., Aleksandrova, Ya.N., and Isaeva, V.V., Stem Cells in Asexual Reproduction of Colonial Ascidian *Botryllus tuberatus, Biol. Morya*, 2007, vol. 33, no. 2, pp. 134–137.

Amikura, R., Kashikawa, M., Nakamura, A., Kobayashi, S., Presence of Mitochondria- Ribosomes Outside Mitochondria in Germ Plasm of *Drosophila* Embryos, *Proc. Natl. Acad. Sci. USA*, 2001, vol. 98, pp. 9133–9138.

Auladell, C., Garcia-Valero, J., and Baguna, J., Ultrastructural Localization of RNA in the Chromatoid Bodies of Undifferentiated Cells (Neoblasts) in Planarians by the RNAse-Gold Complex Technique, *J. Morphol.*, 1993, vol. 216, pp. 319–326.

Beams, H.W. and Kessel, R.G., The Problem of Germ Cell Determinants, *Int. Rev. Cytol.*, 1974, vol. 39, pp. 413–479.

Berrill, N.J., Growth, *Development, and Pattern,* San Francisco: Freeman, 1961.

Bode, H.R., The Interstitial Cell Lineage of Hydra: a Stem Cell System That Arose Early in Evolution, *J. Cell Sci.,* 1996, vol. 109, pp. 1155–1164.

Brown, F.D. and Swalla, B.J., Vasa Expression in a Colonial Ascidians *Botrylloides violaceus, Evol. Devel.*, 2007, vol. 9, pp. 165–177.

Burighel, P. and Cloney, R.A., Urochordata: Ascidiacea, in *Microscopic Anatomy of Invertebrates. Vol. 15*, Harrison, F.W. and Ruppert, E.E., Eds., New York: Wiley-Liss, 1997.

Buss, L.W., Slime Molds, Ascidians and the Utility of Evolutionary Theory, *Proc. Natl. Acad. Sci. USA*, 1999, vol. 96, pp. 8801–8803.

Campbell, R.D., Cnidaria, in *Reproduction of Marine Invertebrates. Vol. 1*, Giese, A.C. and Pierse, J.S., Eds., New York: Academic, 1974.

Carre, D., Djediat, C., and Sardet, C., Formation of a Large Vasa-Positive Germ Granules and Its Inheritance by Germ Cells in the Enigmatic Chaetognaths, *Development*, 2002, vol. 129, pp. 661–670.

Castrillon, D.H., Quade, B.J., Wang, T.Y., et al., The Human VASA Gene Is Specifically Expressed in the Germ Cell Lineage, *Proc. Natl. Acad. Sci. USA*, 2000, vol. 97, pp. 9585–9590.

Chang, C.-C., Lee, W.-C., Cook, C.E., et al., Germplasm Specification and Germline Development in the Parthenogenetic Pea Aphid *Acyrthosiphon pisum*: Vasa and Nanos as Markers, *Int. J. Devel. Biol.*, 2006, vol. 50, pp. 413–421.

Chiquoine, A.D., The Identification, Origin and Migration of the Primordial Germ Cells of the Mouse Embryo, *Anat. Rec.*, 1954, vol. 118, pp. 135–146.

Cima, F., Perin, A., Burighel, P., and Ballarin, L., Morpho-Functional Characterization of Hemocytes of the Compound Ascidian *Botrylloides leachi* (Tunicata, Ascidiacea), *Acta Zool*, 2001, vol. 3, pp. 432–442.

Collins, A.G. and Valentine, J.W., Defining Phyla: Evolutionary Pathways to Metazoan Body Plan, *Evol. Devel.,* 2001, vol. 3, pp. 432–442.

Corley, L.S., White, M.A., and Strand, M.R., Both Eondogenous and Environmental Factors Affect Embryo Proliferation in the Polyembryonic Wasp *Copidosoma floridanum*, *Evol. Devel.*, 2005, vol. 3, pp. 432–442.

Daley, G.Q., Gametes from Embryonic Stem Cells: a Cup Half Empty or Half Full?, *Science*, 2007, vol. 316, pp. 409–410.

Davidson, E.H., Spatial Mechanisms of Gene Regulation in Metazoan Embryos, *Development*, 1991, vol. 113, pp. 1–26.

Davidson, E.H., Peterson, K.J., and Cameron, R.A., Origin of Bilaterian Body Plan: Evolution of Developmental Regulatory Mechanisms, *Science*, 1995, vol. 270, pp. 1319–1325.

Developmental Biology Glossary, (http://home.sandiego.edu/bio176/gloss.html).

Ding, D. and Lipshitz, H.D., Localized RNAs and Their Functions, *BioEssays*, 1993a, vol. 15, pp. 651–658.

Ding, D. and Lipshitz, H.D., A Molecular Screen for Polar Localized Maternal RNAs in the Early Embryo of *Drosophila, Zygote*, 1993b, vol. 1, pp. 257–271.

Donnell, D.M., Corley, L.S., Chen, G., and Strand, M.R., Caste Determination in a Polyembric Wasp Involves Inheritance of Germ Cells, *Proc. Natl. Acad. Sci*. USA, 2004, vol. 101, pp. 10095–10100.

Eddy, E.M., Germ Plasm and the Differentiation of the Germ Cell Line, *Int. Rev. Cytol.*, 1975, vol. 43, pp. 229–280.

Erescovsky, A.V., A New Species of *Oscarella* (Demospongiae: Plakinidae) from the Western Sea of Japan, *Zootaxa*, 2006, no. 1376, pp. 37–51.

Extavour, C.G., The Fate of Isolated Blastomeres with Respect to Germ Cells Formation in the Amphipod Crustacean *Parhyale hawaiensis, Devel. Biol.*, 2005, vol. 277, pp. 387–402.

Extavour, C.G. and Akam, M., Mechanisms of Germ Cell Specification across the Metazoans: Epigenesis and Preformation, *Development*, 2003, vol. 130, pp. 5869–5884.

Extavour, C.G., Pang, K., Matus, D.Q., and Martindale, M.Q., Vasa and Nanos Expression Patterns in a Sea Anemone and the Evolution of Bilaterian Germ Cell Specification Mechanisms, *Evol. Devel.*, 2005, vol. 7, pp. 201–215.

Fujimura, M. and Takamura, K., Characterization of an Ascidian Dead Box Gene, CI-Dead One: Specific Expression in the Germ Cells and Its mRNA Localization in the Posterior Most Blastomeres in Early Embryos, *Devel. Genes Evol.*, 2000, vol. 210, pp. 64–72.

Gilbert, S.F., *Developmental biology*, Sunderland: Sinauer Ass., 2006.

Glossary of Stem Cell-Related Terms, Int. Soc. Stem Cell Res., (http: //*www.isscr.org/glossary*).

Gschwentner, R., Ladurner, P., Nimeth, K., and Rieger, R., Stem Cells in a Basal Bilaterian. S-Phase and Mitotic Cells in *Convolutriloba longifissura, Cell Tiss. Res.*, 2001, vol. 304, pp. 401–408.

Hall, P.A. and Woods A.L., Immunohistochemical Markers of Cellular Proliferation: Achievements, Problems and Prospects, *Cell Tiss. Kinet.*, 1990, vol. 23, pp. 23–55.

Hay, B., Jan, L.Y., and Jan. Y.N., A Protein Component of *Drosophila* Polar Granules Is Encoded by Vasa and Has Extensive Sequence Similarity to ATP Dependent Helicases, *Cell*, 1988, vol. 55, pp. 577–587.

Hayashi, K., de Sousa Lopes, S.M.C., and Surani, M.A., Germ Cell Specification in Mice, *Science*, 2007, vol. 316, pp. 394–396.

Høeg, J.T. and Lützen, J., Life Cycle and Reproduction in the *Cirripedia rhizocephala, Ocean. Mar. Biol. Ann. Rev.*, 1995, vol. 33, pp. 427–485.

Hogan, B., Primordial Germ Cells as Stem Cells, in *Stem Cell Biology*, Marshak, D.R. et al., Eds., New York: Cold Spring Harbor Lab., 2001.

Honegger, T.G., Zurrer, D., and Tardent, P., Oogenesis in *Hydra carnea*: A New Model Based on Light and Electron Microscopic Analyses of Oocyte and Nurse Cell Differentiation, *Tiss.Cell.*, 1989, vol. 21, pp. 381–393.

Hong, Y., Winkler, C., and Schartl, M., Production of Medakafish Chimeras from a Stable Embryonic Stem Cell Line, *Proc. Natl. Acad. Sci. USA*, 1998, vol. 95, pp. 3679–3684.

Hori, I., An Ultrastructural Study of the Chromatoid Body in Planarian Regenerative Cells, *J. Electr. Microsc.*, 1982, vol. 31, pp. 63–72.

Houston, D.W. and King, M.L., Germ Plasm and Molecular Determinants of Germ Cell Fate, *Curr. Top. Devel. Biol.*, 2000, vol. 50, pp. 155–181.

Hubner, K., Fuhrmann, G., Christenson, L., et al., Derivation of Oocytes from Mouse Embryonic Stem Cells, *Science,* 2003, vol. 300, pp. 1251–1256.

Iida, T. and Kobayashi, S. Essential Role of Mitochondrially Encoded Large rRNA for Germline Formation in *Drosophila* Embryos, *Proc. Natl. Acad. Sci. USA*, 1998, vol. 95, pp. 11274–11278.

Ikenishi, K., Germ Plasm in *Caenorabditis elegans, Drosophila* and *Xenopus, Devel. Growth. Differ.*, 1998, vol. 40, no. 1, pp. 1–10.

Isaeva, V.V. and Shukalyuk, A.I., *Kolonial'nye kornegolovye rakoobraznye (Crustacea: Rhizocerhala): bespoloe razmnozhenie, stvolovye kletki, reproduktivnaya strategiya* (Colonial Rhizocephalans (Crustacea: Rhizocerhala): Asexual Reproduction, Stem Cells, and Reproductive Strategy), Moscow: Nauka, 2007.

Isaeva, V.V., Rybakov, A.V., and Kas'yanov, V.L., *In vitro* Establishment of the Colonial Organization of the Interna of the Rhizocephalan Crustaceans *Peltogasterella gracilis* and *Sacculina polygenea, Dokl. Akad. Nauk*, 1999, vol. 366, no. 6, pp. 840–842.

Isaeva, V.V., Shukalyuk, A.I., Trofimova, A.V., et al., The Structure of Colonial Interna in *Sacculina polygenea* (Crustacea: Cirrlpedia: Rhizocephala), *Crust. Res.*, 2001a, no. 30, pp. 134–147.

Isaeva, V.V. and Reunov, A.A., Germ Plasm and Germ Cell Line Determination: Role of Mitochondria, *Biol. Morya*, 2001b, vol. 27, no. 4, pp. 231–237.

Isaeva, V.V., Shukalyuk, A.I., and Kizilova, E.A., Recognition of Stem Cells in the Colonial Internae of the Rhizocephala *Peltogasterella gracilis* and *Sacculina polygenea* at the Parasitic Life Cycle Stage, *Tsitologiya*, 2003, vol. 45, no. 8, pp. 758–763.

Isaeva, V.V., Shukalyuk, A.I., Korn, O.M., and Rybakov, A.V., Development of Primordial Externae in the Colonial Interna of Polyascus Polygenea (Crustacea: Cirripedia: Rhizocephala), *Crust. Res.*, 2004, no. 33, pp. 61–71.

Isaeva, V., Alexandrova, Ya., and Reunov, A., Interaction between Chromatoid Bodies and Mitochondria in Neoblasts and Gonial Cells of the Asexual and Spontaneously Sexualized Planarian *Girardia (Dugesia) tigrina, Invertebr. Reprod. Devel.*, 2005, vol. 48, pp. 119–128.

Isaeva, V.V., Shukalyuk, A.I., and Akhmadieva, A.V., Stem Cells in Reproductive Strategy of Asexually Reproducing Invertebrates, *Biol. Morya*, 2007, vol. 33, no. 1, pp. 3–10.

Isaeva, V.V., Shukalyuk, A.I., and Akhmadieva, A.V., Asexual Reproduction and Reproductive Strategy in Rhizocephalans (Crustacea: Cirripedia: Rhizocerhala), *Zool. Zh.*, 2008, vol. 87, no. 3, pp. 1–12.

Ivanova-Kazas, O.M., *Bespoloe razmnozhenie zhivotnykh* (Asexual Reproduction of Animals), Leningrad: Leningr. Gos. Univ., 1977.

Ivanova-Kazas, O.M., *Sravnitel'naya embriologiya bespozvonochnykh zhivotnykh. Nizshie khordovye* (Comparative Embryology of Invertebrates. Lower Chordates), Moscow: Nauka, 1978.

Ivanova-Kazas, O.M., *Sravnitel'naya Embriologiya Bespozvonochnykh Zhivotnykh. Chlenistonogie*, (Comparative Embryology of Invertebrates. Arthropods), Moscow: Nauka, 1979.

Ivanova-Kazas, O.M., *Sravnitel'naya Embriologiya Bespozvonochnykh Zhivotnykh. Nepolnousye* (Comparative Embryology of Invertebrates. Atelocerata), Moscow: Nauka, 1981.

Jenner, R.A., Evolution of Animal Body Plans: the Role of Metazoan Phylogeny at the Interface between Pattern and Process, *Evol. Devel.*, 2000, vol. 2, pp. 208–221.

Juliano, C.E., Voronina, E., Stack, C., et al., Germ Line Determinants Are not Localized Early in Sea Urchin Development, but do Accumulate in the Small Micromere Lineage, *Devel. Biol.*, 2006, vol. 300, pp. 406–415.

Kashikawa, M., Amikura, R., Nakamura, A., and Kobayashi, S., Mitochondrial Small Ribosomal RNA Is Present on Polar Granules in Early Cleavage Embryos of *Drosophila melanogaster, Devel. Growth Differ.*, 1999, vol. 41, pp. 495–502.

Kasyanov, V.L., *Reproduktivnaya strategiya morskikh dvustvorchatykh mollyuskov i iglokozhikh* (Reproductive Strategy of Marine Bivalve Molluscs and Echinodermes), Leningrad: Nauka, 1989.

Kasyanov, V.L., *Reproductive Strategy of Marine Bivalves and Echinoderms*, Enfield: Science Publishers, 2001.

Kas'yanov, V.L., Korn, O.M., and Rybakov, A.V., Reproductive Strategy of Barnacles (Thecostraca, Cirripedia). 1. Sexual Dimorphism, Reproductive System, Gametogenesis, *Biol. Morya*, 1997a, vol. 23, no. 5, pp. 263–274.

Kas'yanov, V.L., Korn, O.M., and Rybakov, A.V., Reproductive Strategy of Cirripedes (Thecostraca, Cirripedia). 2. Asexual Reproduction, Fecundity and Reproductive Cycles, *Biol. Morya*, 1997b, vol. 23, no. 6, pp. 337–344.

Kas'yanov, V.L., Korn, O.M., and Rybakov, A.V., Reproductive Strategy of Cirripedes (Thecostraca, Cirripedia). 3. Embryonic Development and Early Larvae, *Biol. Morya*, 1998, vol. 24, no. 5, pp. 269–277.

Kas'yanov, V.L., Korn, O.M., and Rybakov, A.V., Reproductive Strategy of Cirripedes (Thecostraca, Cirripedia). 4. Cypris Larvae, Metamorphosis and Settlement, *Biol. Morya,* 1999, vol. 25, no. 1, pp. 3–12.

Kerkis, A., Fonseca, S.A.S., Serafim, R.C., et al., *In vitro* Differentiation of Male Mouse Embryonic Stem Cells into Both Presumptive Sperm Cells and Oocytes, *Cloning Stem Cells*, 2007, vol. 9, no. 4, pp. 535–548.

Kerszberg M. and Wolpert, L., The Origin of Metazoa and The Egg: a Role for Cell Death, *J. Theor. Biol*, 1998, vol. 193, pp. 535–537.

Klenov, M.S., Stolyarenko, A.D., Ryazanskii, S.S., et al., Role of Short RNAs in Regulating the Expression of Genes and Mobile Elements in Germ Cells, *Ontogenez*, 2007, vol. 38, no. 3, pp. 213–227.

Kloc, M., Bilinski, S., Chan, A.P., and Etkin, L.D., Mitochondrial Ribosomal RNA in the Germinal Granules in *Xenopus* Embryos Revisited, *Differentiation*, 2000, vol. 67, pp. 80–83.

Kobayashi, S. and Okada, M., Restoration of Pole Cell Forming Ability to U.-V.-Irradiated *Drosophila* Embryos by Injection of Mitochondrial lrRNA, *Development*, 1989, vol. 107, pp. 733–742.

Kobayashi, S., Amikura, R., and Okada, M., Presence of Mitochondrial Large Ribosomal RNA outside Mitochondria in Germ Plasm of *Drosophila melanogaster*, *Science*, 1993, vol. 260, pp. 1521–1524.

Kobayashi, S., Amikura, R., and Mukai, M., Localization of Mitochondrial Large Ribosomal RNA in Germ Plasm of *Xenopus* Embryos, *Curr. Biol.*, 1998, vol. 8, pp. 1117–1120.

Kobayashi, S., Sato, K., and Hayashi, Y., The Role of Mitochondrial rRNAs and Nanos Protein in Germline Formation in *Drosophila* Embryos, *Zool. Sci.*, 2005, vol. 22, pp. 943–954.

Kotaja, N., Bhattacharyya, S.N., Jaskiewicz, L., et al., The Chromatoid Bodies of Male Germ Cells: Similarity with Processing Bodies and Presence of Dicer and microRNA Pathway Components, *Proc. Natl. Acad. Sci. USA*, 2006, vol. 103, pp. 2647–2652.

Kozin, V.V., Smirnova, N.P., Babakhanova, R.A., and Kostyuchenko, R.P., Cell Sources of Growth Zone Development in Annelids during Metamorphosis, Regeneration, and Asexual Reproduction, in *Mater. simp. "Kletochnye, molekulyarnye i evolyutsionnye aspekty morfogeneza"* (Proc. Symp. Cellular, Molecular, and Evolutionary Aspects of Morphogenesis), Moscow: Tov-vo nauch. izdanii KMK, 2007, pp. 85–87.

Kunwar, P.S. and Lehmann, R., Developmental Biology: Germ Cell Attraction, *Nature*, 2003, vol. 421, pp. 226–227.

Lacham-Kaplan, O., *In Vivo* and *In Vitro* Differentiation of Male Germ Cells in the Mouse, *Reproduction*, 2004, vol. 128, pp. 147–152

Laird, D.J. and de Tomaso, A.W., Predatory Stem Cells in the Non-Zebrafish Chordate, *Botryllus schlosseri, Zebrafish*, 2004/2005, vol. 1, pp. 357–361.

Laird, D.J. and Weissman, I.L., Telomerase Maintained in Self-Renewing Tissues during Serial Regeneration of the Urochordate *Botryllus schlosseri, Devel. Biol.*, 2004, vol. 273, pp. 185–194.

Laird, D.J., de Tomaso, A.W., and Weissman, I.L., Stem Cells are Units of Natural Selection in a Colonial Ascidian, *Cell*, 2005, vol. 123, pp. 1351–1360.

Leatherman, J.L. and Jongens, T.A., Transcriptional Silencing and Translational Control: Key Features of Early Germline Development, *BioEssays*, 2003, vol. 25, pp. 326–335.

Lim, A.K. and Kai, T., Unique Germline Organelle, Nuage, Functions to Repress Selfish Genetic Elements in *Drosophila melanogaster, Proc. Natl. Acad. Sci. USA*, 2007, vol. 104, pp. 6714–6719.

Mahowald, A.P., Polar Granules in *Drosophila.* III. The Continuity of Polar Granules during the Life Cycle of *Drosophila, J. Exp. Zool.*, 1971, vol. 170, pp. 551–563.

Mahowald, A.P., Assembly of the *Drosophila* Germ Plasm, *Int. Rev. Cytol.*, 2001, vol. 203, pp. 187–213.

Matova, N. and Cooley, L., Comparative Aspects of Animal Oogenesis, *Devel. Biol.*, 2001, vol. 16, pp. 1–30.

Merchant-Larios, H., Mendelovic, F., and Alvarez-Buyalla, A., Characterization of Alkaline Phosphatase from Primordial Germ Cells and Ontogenesis of This Enzyme in the Mouse, *Differentiation*, 1985, vol. 29, pp. 145–151.

Mintz, B., Continuity of the Female Germ Cell Line from Embryo to Adult, *Arch. Micr. Morphol. Exp.*, 1959, vol. 48, pp. 155–172.

Mochizuki, K., Nishimiya-Fujisawa, C., and Fujisawa, T., Universal Occurrence of the Vasa Related Genes among Metazoans and Their Germline Expression in *Hydra, Devel. Genes Evol.,* 2001, vol. 211, pp. 299–308.

Mueller, T. and Wullimann, M.F., Anatomy of Neurogenesis in the Early Zebrafish Brain, *Devel. Brain Res.*, 2003, vol. 140, pp. 137–155.

Müller, W.E.G., Telomerase Activity in Sponges (Porifera) the Closest Related Taxa of the Hypothetical Ancestral Animal the Urmetazoa, in *Telomeres and Telomerase*, Krupp, G. and Parwaresch, R., Eds., Georgetown: Mol. Biol. Intelligence, 2002, pp. 300–313.

Müller, W.E.G., The Stem Cell Concept in Sponges (Porifera): Metazoan Traits, *Sem. Cell Devel. Biol.,* 2006, vol. 17, pp. 481–491.

Nikitin, N.S., Morphogenetic Potential of Clusters of Nuclear Amoebocytes in Freshwater Sponge *Erhudatia fluviatilis* L. as a Function of Their Size, in *Morfogeneticheskie protsessy pri raznykh tipakh razmnozheniya i v khode regulyatsii* (Morphogenetic Processes in Different Reproduction Types and Under Control), Tokin, B.P., Ed., Leningrad: Leningr. Gos. Univ., 1974.

Noda, K. and Kanai, C., An Ultrastructural Observation of *Pelmatohydra robusta* at Sexual and Asexual Stages, with a Special Reference to "Germinal Plasm," *J. Ultrastruct. Res.*, 1977, vol. 61, pp. 284–294.

Orii, H., Sakurai, T., and Watanabe, K., Distribution of the Stem Cells (Neoblasts) in the Planarian *Dugesia japonica, Devel. Genes Evol.*, 2005, vol. 215, pp. 143–157.

Pain, B., Clark, M.E., Shen, M., et al., Long-Term *In vitro* Culture and Characterization of Avian Embryonic Stem Cells with Multiple Morphogenetic Potentialitiesm, *Development,* 1996, vol. 122, pp. 2339–2348.

Pancer, Z., Gershon, H., and Rinkevich, B., Coexistence and Possible Parasitism of Somatic and Germ Cell Lines in Chimeras of the Colonial Urochordate *Botryllus schlosseri, Biol. Bull.*, 1995, vol. 189, pp. 106–112.

Pesce, M., Anastassiadis, K., and Scholer, H.R., Oct-4: Lessons of Totipotency from Embryonic Stem Cells, *Cells Tiss. Organs*, 1999, vol. 165, pp. 144–152.

Peter, R., Ladurner, P., and Rieger, R.M., The Role of Stem Cell Strategies in Coping with Environmental Stress and Choosing between Alternative Reproductive Modes: Turbellaria Rely on a Single Cell Type to Maintain Individual Life and Propagate Species, *Marine Ecol.*, 2001, vol. 22, no. 1–2, pp. 35–51.

Peterson, K.J., Cameron, R.A., and Davidson, E.H., Set-Aside Cells in Maximal Indirect Development: Evolutionary and Developmental Significance, *BioEssays*, 1997, vol. 19, pp. 623–631.

Raz, E., The Function and Regulation of the Vasa like Genes in Germ Cell Development, *Genome Biol.*, 2000, vol. 3, pp. 1017.1–1017.6.

Reddy, S., Rayburn, H., von Melchner, H., and Ruley, HE., Fluorescence Activated Sorting of Totipotent Embryonic Stem Cells Expressing Developmentally Regulated LacZ Fusion Genes, *Proc. Natl. Acad. Sci. USA*, 1992, vol. 89, pp. 6721–6725.

Reunov, A., Isaeva, V., Au, D., and Wu, R., Is It Possible the Arising of Nuage Constituents from Mitochondria? *Devel. Growth Differ.*, 2000, vol. 42, pp. 129–143.

Reunov, A.A., Neznanova, S.Yu., Aleksandrova, Ya.N., and Isaeva, V.V., An Ultrastructural Study of the Interaction of Germinal Granules and Mitochondria in *Apostichopus japonicus* (Echinodermata: Holothuroidea) and *Pleuronectes*

asper (Teleostei: Pleuronectidae), *Biol. Morya*, 2004, vol. 30, no. 3, pp. 244–246.

Rieger, R.M., Tyler, S., Smith, J.P.S. III, and Rieger, G.E., Plathelminthes: Turbellaria, in *Microscopic Anatomy of Invertebrates, vol. 3. Plathelminthes and Nemertea*, Harrison, F.W., Ed., New York: Wiley-Liss, 1991.

Rybakov, A.V. and Shukalyuk, A.I., *Thylacoplethus isaevae* sp. n., a New Species of Colonial Rhizocephalans (Crustacea: Rhizocephala: Thompsoniidae) Parasitic on a Northwest Pacific Hermit Crab *Pagurus trigonocheirus* (Stimpson), *J. Marine Biol. Ass. UK*, 2004, vol. 84, pp. 1009–1017.

Saffman, E.E. and Lasko, F., Germline Development in Vertebrates and Invertebrates, *Cell Mol. Life Sci.,* 1999, vol. 55, pp. 1141–1163.

Saitou, M., Payer, B., Lange, U.C., et al., Specification of Germ Cell Fate in Mice, *Phil. Trans. Royal Soc. L.*, 2003, vol. 358, pp. 1363–1370.

Sato, K., Sugita, T., Kobayashi, K., et al., Localization of Mitochondrial Ribosomal RNA on the Chromatoid Bodies of Marine Planarian Polyclad Embryos, *Devel. Growth Differ.*, 2001, vol. 43, pp. 107–114.

Seydoux, G. and Braun, R.E., Pathway to Totipotency: Lessons from Germ Cells, *Cell*, 2006, vol. 127, pp. 891–904.

Shibata, N., Umesono, Y., Orii, H., et al., Expression of Vasa (Vas)-Related Genes in Germline Cells and Totipotent Somatic Stem Cells of Planarians, *Devel. Biol.*, 1999, vol. 206, pp. 73–87.

Shukalyuk, A.I. and Isaeva, V.V., Stem Cells in Invertebrate Animals: Evolutionary Conservative Mechanism of Totipotency, *Int. Soc. Stem Cell Res. San Francisco*, 2005a, pp. 214–215.

Shukalyuk, A.I., Isaeva, V.V., Stem and Germ Cells in Asexually Reproducing Animals: a Comparative Study, *Mol. Biol. Cell.*, 2005b, vol. 16, no. 11 (Suppl.), pp. 752a.

Shukalyuk, A., Isaeva, V., Kizilova, E., and Baiborodin, S., Stem Cells in Reproductive Strategy of Colonial Rhizocephalan Crustaceans (Crustacea: Cirripedia: Rhizocephala), *Invertebr. Reprod. Devel.*, 2005, vol. 48, pp. 41–53.

Shukalyuk, A.I., Golovnina, K.A., Baiborodin, S.I., et al., Vasa Related Genes and Their Expression in Stem Cells of Colonial Parasitic Rhizocephalan Barnacle *Polyascus polygenea* (Crustacea: Cirripedia: Rhizocephala), *Cell Biol. Int.*, 2007, vol. 31, pp. 97–108.

Simpson, T.L., The Cell Biology of the Sponges. New York: Springer-Verlag, 1984.

Smith, A., Embryonic Stem Cells, in *Stem Cell Biology*, Marshak, D.R. et al., Eds., New York: Cold Spring Harbor Lab., 2001.

Smith, A., A Glossary for Stem Cell Biology, *Insight Nature*, 2006, vol. 441, pp. 1060. (http://*www.grg.org/resourses/ASmithStemCellGlossary.htm)*

Stem Cell Biology, Marshak, D.R. et al., Eds., New York: Cold Spring Harbor Lab., 2001.

Stem Cell Information. The National Institutes of Health Resource for Stem Cell Research. (http://*stemcells.nih.gov/info/scireport/appendixF.asp*)

Stoner, D.S. and Weissman, I.L., Somatic and Germ Cell Parasitism in a Colonial Ascidian: Possible Role for a Highly Polymorphic Allorecognition System, *Proc. Natl. Acad. Sci. USA*, 1996, vol. 93, pp. 15254–15259.

Stoner, D.S., Rinkevich, B., and Weissman, I.L., Heritable Germ and Somatic Cell Lineage Competitions in Chimeric Colonial Protochordates, *Proc. Natl. Acad. Sci. USA*, 1999, vol. 96, pp. 9148–9153.

Stoop, H., Honecker, F., Cools, M., et al., Differentiation and Development of Human Female Germ Cells during Prenatal Gonadogenesis: an Immunohistochemical Study, *Hum. Reprod.,* 2005, vol. 20, pp. 1466–1476.

Strome, S. and Lehman, R., Germ Versus Soma Decisions: Lessons from Flies and Worms, *Science*, 2007, vol. 316, pp. 392–393.

Sunanaga, N., Saito, Y., and Kawamura, K., Postembryonic Epigenesis of Vasa Positive Germ Cells from Aggregated Hemoblasts in the Colonial Ascidian, *Botryllus primigenus, Devel. Growth Differ.*, 2006, vol. 48, pp. 87–100.

Takahashi, T. and Lützen, J., Asexual Reproduction as Part of the Life Cycle in *Sacculina polygenea* (Cirripedia: Rhizocephala: Sacculinidae), *J. Crust Biol.*, 1998, vol. 18, no. 2, pp. 321–331.

Takamura, K., Fujimura, M., and Yamaguchi, Y., Primordial Germ Cells Originate from the Endodermal Strand Cells in the Ascidian *Ciona intestinalis, Devel. Genes Evol.*, 2002, vol. 212, pp. 11–18.

Talbot, N.C., Rexroad, C.E. Jr., Pursel, V.G., and Powell, A.M., Alkaline Phosphatase of Pig and Sheep Epiblast Cells in Culture, *Mol. Reprod. Devel.*, 1993, vol. 36, pp. 139–147.

Teragawa, C.K. and Bode, H.R., Spatial and Temporal Patterns of Interstitial Cell Migration in *Hydra vulgaris, Devel. Biol.*, 1990, vol. 138, pp. 63–81.

Thomas, M.B. and Edwards, N.C., Cnidaria: Hydrozoa, *Microsc. Anat. Invertebr.*, 1991, vol. 2, pp. 91–183.

Travis, J., A Close Look at Urbisexuality, *Science*, 2007, vol. 316, pp. 390–391.

Villegas, J., Araya, P., Bustos-Obregon, E., and Burzio, L.O., Localization of the 16S Mitochondrial rRNA in the Nucleus of Mammalian Spermatogenic Cells, *Mol. Hum. Reprod.,* 2002, vol. 8, pp. 977–983.

Watanabe, M., Itoh, K., Abe, K., et al., Immuno Localization of Dead Family Proteins in Germ Cell Line Cells of *Xenopus* embryos, *Devel. Growth Differ.*, 1992, vol. 34, pp. 223–231.

Weismann, A., Das Keimplasma. *Eine Theorie der Vererbung*, Jena: Verlag von Gustav Fisher, 1882.

Weismann, A., *The Germ Plasm. A Theory of Heredity*, New York: Charles Scriber's Sons, 1893.

Weissman, I.L., Stem Cells: Units of Development, Units of Regeneration, and Units in Evolution, *Cell*, 2000, vol. 100, pp. 157–168.

Williamson, A. and Lehman, R., Germ Cell Development in *Drosophila, Annu. Rev. Devel. Biol.*, 1996, vol. 12, pp. 365–391.

Winslow, T., Stem cells. Scientific Progress and Future Research Directions, Bethesda: Nat. Inst. Health, 2001.

Wolpert, L., Stem Cells: a Problem in Asymmetry, *J. Cell Sci.*, 1988, vol. 10 suppl., pp. 1–9.

Wylie, C., Germ Cells, *Cell*, 1999, vol. 96, pp. 165–174.