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# Pluripotent Gametogenic Stem Cells of Asexually Reproducing Invertebrates

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## 1. Introduction

In this review, morphological and some functional properties of stem cells in different representatives of animals with asexual reproduction (sponges, hydroids, planaria, colonial rhizocephalan crustaceans and colonial ascidia) are considered in comparison with metazoan germline cells and *in vitro* mammalian embryonic stem cells.

Stem cells are an essential and defining feature developed during evolution of all multicellular organisms (Lohman, 2008; Batygina, 2010; Funayama et al., 2010). The study of mammalian embryonic stem cells has become a hot, intensely developing field in biology, biotechnology, and biomedicine. However, comparative studies of stem cells in various multicellular organisms are required to understand molecular mechanisms of maintaining pluri/totipotency, “stemness”, which still remain far from clear, as well as mechanisms that regulate gametogenesis, reproduction, development and regeneration.

According to the generally accepted view, stem cells are cells of embryos or adult organisms capable of self-renewing by mitotic reproduction and differentiation into specialized cell types (Weissman et al., 2001; Cogle et al 2003; Müller, 2006; Lohman 2008; Rinkevich et al., 2009; Sköld et al., 2009; Funayama et al., 2010).

Two main types of stem cells should be considered: the cells of the germline and the somatic stem cell lineages (Hogan, 2001; Rinkevich, 2009; Strouji & Extavour, 2011). Germ cells are the only cell type capable of generating a new whole organism in animals; everlasting germline cycle continues from one generation to the next, thus the germline escapes the mortality that all somatic cells of an organism ultimately confront (the common view from Weismann, 1892, to Cinalli et al., 2008). The true innovation in the evolution was the generation of gametogenic germ line, the loss of gametogenic potential from the majority of cells of the organism and protection of the germ line throughout development (Extavour, 2008; Strouji & Extavour, 2011). During development, germ cells are set aside from all somatic cells of the embryo (Cinalli et al., 2008).

E. Davidson and colleagues have developed the “set-aside” hypothesis (Davidson et al., 1995; Cameron et al., 1998; Jenner, 2000; Collins & Valentine, 2001). Cameron et al. (1998) called “set-aside cells” the patches of larval cells that give rise to the adult body plan in animals with indirect development while most of the larval cells have a dead-end fate. These cells are developmentally set aside from the embryo-larva differentiation process,

have an essentially unlimited division capacity, and they produce new populations of cells that are organized into the parts of the adult body plan (Cameron et al., 1998). So many adult organs are not derived from cells within larval organs, but from pluripotent cells sequestered, set aside during larval life as primordia from which adult structures form, such as imaginal discs of insects (Collins & Valentine, 2001).

Generally, we can regard all stem cells as “set-aside cells”, reserve cells.

The reproductive strategy of multicellular organisms can include sexual and asexual reproduction. In organisms with exclusively sexual reproduction, primary germ cells become segregated during embryogenesis. Two main types of germline cell specification were defined as preformation (early specification of primary germ cells by means of asymmetric distribution of maternal cytoplasmic determinants) and epigenesis, i.e. later specification of germ lineage cells by inductive signals (Extavour & Akam, 2003; Travis, 2007; Frank et al., 2009). Recently, Strouji and Extavour (2011) refer to former “preformation” as “inheritance mode”, and former “epigenesis” as “inductive mode”. Besides, a third variant of germ cell specification, along with preformation and epigenesis, is recognized as somatic embryogenesis, in asexually reproducing animals, which have stem cells able to differentiate into germ and somatic cells throughout the life of an individual or a colony (Buss, 1987, 1999; Blackstone & Jasker, 2003; Frank et al., 2009; Rinkevich et al., 2009). Pluri/totipotent stem cells of these animals provide a cell source for gametogenesis, asexual reproduction and regeneration (Isaeva et al., 2008b, 2009; Frank et al., 2009; Sköld et al., 2009). Stem cells that have the potential to become both somatic and primary germ cells and are morphologically indistinguishable from the latter were defined as “primary stem cells” (Sköld et al., 2009).

In animals with asexual reproduction by somatic embryogenesis, the germ lineage remains non-segregated even in the adult organism, the gametes of which differentiate from stem cells (Blackstone & Jasker, 2003; Rinkevich, 2009; Sköld et al., 2009). Examples of such reserve stem cells, capable of differentiating both into germ and somatic cells, include sponge archaeocytes, cnidarian interstitial cells, planarian neoblasts, and stem cells of colonial ascidians (reviews: Isaeva et al., 2008b, 2009; Frank et al., 2009; Rinkevich et al., 2009; Sköld et al., 2009; Isaeva, 2010; Strouji, Extavour, 2011). These self-renewing stem cells maintains continuously throughout the life of an individual or a colony, being predecessors of germ cells and all the types (or a wide spectrum) of somatic cells, so ensuring both sexual and asexual reproduction. Thus, in invertebrates with asexual reproduction, no early segregation of a germ cell lineage takes place, and a self-renewing reserve of stem cells with broad or unlimited morphogenetic potential is maintained over the entire lifespan.

Similarly in plants, floral stem cells arise from stem cells of shoot apical meristema (Verdeil et al., 2007; Lohman, 2008; Batygina, 2010). Somatic embryogenesis is viewed as a condition characteristic to the lower metazoans; in the course of evolution, metazoans switch from somatic embryogenesis to preformation and epigenesis, and a subsequent return to somatic embryogenesis is a rare event (Blackstone & Jasker, 2003). If epigenesis was used by Urbilateria to specify the germ line, then preformation must have evolved convergently several times during the bilaterian radiation (Extavour, 2008).

In organisms with asexual, agamous development, the organism that has developed from the zygote is capable of natural cloning and forming numerous genetically identical individuals or modular units of a colony. Clonal morphogenesis, called somatic embryogenesis, as applied to animals, was usually termed blastogenesis (Berrill, 1961; Ivanova-Kazas, 1996) while in plants it was termed somatic embryogenesis or

embryoidogenesis (see Verdeil et al., 2007; Batygina, 2010). In the life cycle of colonial animals, one generation of oozoid (individual that has developed from an egg) alternates with numerous generations of blastozooids, with respectively alternating morphogenetic processes: embryogenesis and blastogenesis (Ivanova-Kazas, 1996). In animal kingdom, natural cloning is a widespread phenomenon that includes polyembryony, budding, fission (architomy, paratomy, autotomy) etc.

Both striking similarities and considerable differences in stem cell systems have been observed between plants and animals (Lohman, 2008; Batygina, 2010; Sablowski, 2010). Lohman (2008) considers as the most important differences the capacity of plants to maintain totipotent stem cells throughout their entire lives and the dramatic developmental plasticity of plant cells; besides, plant cells are unable to move within the organism by active migration. Similarities in the stem cell pools of plants and animals suggest that there might have been strong evolutionary constraints that shaped the path for the development of stem cell systems (Lohman, 2008; Sablowski, 2010).

Our study points towards elucidation of the evolutionary conservative cellular, sub-cellular and molecular bases of “stemness”, focusing on the comparative investigation of pluripotent stem cells in reproducing asexually representatives of five metazoan types: archaeocytes in the sponge *Oscarella malakhovi* (Porifera), interstitial cells in the colonial hydroids *Obelia longissima* and *Ectopleura crocea* (Cnidaria), neoblasts in the planarian *Girardia (Dugesia) tigrina* (Plathelminthes), stem cells in the colonial parasitic rhizocephalan crustaceans *Peltogasterella gracilis* and *Polyascus polygenea* (Arthropoda), the colonial ascidian *Botryllus tuberatus* (Chordata), and also mammalian embryonic stem cells as a benchmark, “standard reference”, using *in vitro* culture, electron microscopic, histological, some histochemical, immunochemical, and molecular methods. Our hypothesis is the evolutionary conserved structural and functional organization of pluri/totipotent cells with gametogenic potentiality and of the molecular mechanisms maintaining it, in embryonic stem, reserve “primary” stem, and germline cells of different metazoan animals. Germplasm granules (or nuage) were studied as an ultrastructural marker and key organelle of germline cells and pluripotent stem cells of invertebrates. We have chosen as a molecular marker the evolutionarily conserved members of DEAD-box family (*vasa* and *pl10* related genes) whose expression was revealed by other researchers in germinal granules of germline cells in various metazoan taxa (see below). The reaction revealing the activity of alkaline phosphatase, formerly used for the identification of embryonic stem and primary germ cells of mammals and other vertebrate animals, was applied as a cytochemical marker of the pluripotent cells of invertebrates. The proliferating cell nuclear antigen, PCNA, was used as a marker of the proliferation of self-renewing stem cells.

Sponge archaeocytes, cnidarian interstitial cells, and planarian neoblasts are classical, long-explored stem cells. Rhizocephalan crustaceans (Arthropoda: Crustacea: Cirripedia: Rhizocephala) parasitizing free-living crustaceans, mainly decapods, until now are often considered as incapable of asexual reproduction and coloniality (for example, Blackstone & Jasker, 2003; Sköld et al., 2009). However, at the parasitic stage of the life cycle, many species of rhizocephalan have asexual reproduction without separation of blastozooids, resulting in the emergence of colonial organization (Høeg, 1992; Høeg & Lützen, 1995; Isaeva et al., 2003, 2004; Shukalyuk et al., 2005, 2007, 2011) that is unique among crustaceans, all arthropods, and all Ecdysozoa. Direct evidence of colonial organization at the parasitic stage of life cycle has been obtained only for a few rhizocephalan species. We visualized asexual reproduction, revealed and studied stem cells in the stolons and buds of the colonial

rhizocephalans *Polyascus polygenea* and *Peltogasterella gracilis* (Isaeva et al., 2003, 2004; Shukalyuk et al., 2005, 2007). Hemoblasts of the colonial ascidians *Botryllus schlosseri*, *Botrylloides leachi* and other species are considered as putative totipotent or pluripotent stem cells (Pancer et al., 1995; Burighel & Cloney, 1997; Stoner et al., 1999; Cima et al., 2001; Laird et al., 2005; Sunanaga et al., 2006), although stem cells have not been morphologically identified in the genus *Botryllus*. We have found stem cells, morphologically similar to hemoblasts in *B. leachi* (Cima et al., 2001) and other studied ascidians (Burighel & Cloney, 1997), in the early buds and vascular system of the colonial ascidian *Botryllus tuberatus* (Akhmadiyeva et al., 2007).

The data of our team showed that studied pluripotent reserve stem cells serve as the predecessors of germ and somatic cells and display some evolutionarily conserved features of the morphological and functional organization typical also for cells of the germ line and embryonic stem cells (Isaeva et al., 2003, 2004, 2005, 2008b, 2009, 2011; Akhmadieva et al., 2007; Shukalyuk, Isaeva, 2005; Shukalyuk et al., 2005, 2007, 2011; Isaeva, 2010; Isaeva, Akhmadieva, 2011).

## **2. Stem cells in asexually reproducing animals share common features with germ cells and embryonic stem cells**

### **2.1 Pluri/totipotency**

In animals with asexual reproduction, the differentiation of “primary” stem cells into germ and somatic cells is delayed, the germ lineage in these animals is not segregated (Tuzet, 1964; Buss, 1987, 1999; Sköld et al., 2009); these stem cells serve as the cellular source of asexual and sexual reproduction as well as of regeneration (Isaeva et al., 2008b, 2009; Frank et al., 2009). Pluripotent stem cells can differentiate into a very wide spectrum of somatic cells in adult organisms.

Depending on the width of the potential range of cell differentiation, totipotent, pluripotent, multipotent, oligopotent, and unipotent stem cells are distinguished, but the usage of this terminology is not unified (Müller, 2006; Newton, 2006; Isaeva et al., 2008b, 2009; Rinkevich et al., 2009; Sköld et al., 2009). Totipotent cells can give rise to all cell types of a developing organism; the zygote and the cells of the early mammalian embryo are totipotent. Stem cells of invertebrates reproducing asexually are traditionally often but not always referred to as totipotent, if their ability to differentiate into gametes and all somatic cells of the organism is shown (reviews: Isaeva et al., 2008b, 2009; Rinkevich et al., 2009; Sköld et al., 2009). For instance, archaeocytes of sponges are regarded as totipotent (Simpson, 1984; Müller, 2006) or pluripotent (Funayama, 2008; Funayama et al., 2010). Funayama believes that both archaeocytes and choanocytes of sponges are pluripotent. Neoblasts of planarians are considered totipotent (Shibata et al., 1999; Gschwentner et al., 2001; Peter et al., 2001; Sköld et al., 2009) or pluripotent (Shibata et al., 2010). Similarly, stem cells of colonial rhizocephalan crustaceans can also be considered totipotent (Isaeva et al., 2004, 2008b, 2009; Shukalyuk et al., 2005, 2007) or pluripotent. The stem cells in colonial ascidians are named both totipotent and pluripotent cells (Stoner et al., 1999; Weissman, 2000; Laird & Weissman, 2004; Laird et al., 2005; Sunanaga et al., 2006). Stem cells giving rise to germline and many but not all somatic cell types are referred to as multipotent. Cnidarian interstitial cells are usually believed to be multipotent, especially in the genus *Hydra* (Bode, 1996; Mochizuki et al., 2001; Frank et al., 2009), but the interstitial cells of *Hydractinia echinata* are recognized as totipotent (Frank et al., 2009).

Estimations of the potentiality of female germ line cells are also contradictory: such cells can be qualified as unipotent, since they produce only one type of differentiated cells, and totipotent, taking into account their potential of developing into a whole organism (Hogan, 2001; Seydoux & Braun, 2006; Strome & Lehman, 2007). Nussbaum (1880) recognized the germ line cells as totipotent and principally different from somatic cells with their limited potency. Accepting the concept of the maintenance of totipotency by cells of the female germ lineage, the author believes that the ability of the stem cells in asexually reproducing invertebrates to differentiate into female gametes and potentially to develop into a whole organism gives grounds for considering them as totipotent independently of the width of their somatic derivatives (Isaeva et al., 2008b, 2009).

If we understand cell totipotency as the ability of a single cell to produce a whole organism, only the zygote and the blastomeres of the early embryo of mammals and other animals with regulative type of development are totipotent, having the potential to form an entire living organism. Mammalian embryonic stem cells derived from inner cell mass of the blastocyst have pluripotency: they are able to differentiate into tissues of all three germ layers but cannot produce a whole embryo (Cogle et al. 2003). On the other hand, mammalian embryonic stem cells are able to give oogenic cells and oocytes entering meiosis and parthenogenetically producing blastocyst-like cell masses (Hübner et al., 2003; Daley, 2007; Kerkis et al., 2007).

In plants, a new individual can develop from one totipotent somatic cell (Verdeil et al., 2007; Lohman, 2008; Batygina, 2010; Sablowski, 2010). In the asexual reproduction of plants, for instance in polyembryony, a new individual develops from one stem cell, and the pattern of the cell divisions is similar to the cleavage of the zygote (Batygina, 2010). Thus, a single totipotent stem cell of plants may be similar to zygote. The ability of plants to maintain totipotent stem cells over the entire life span of the organism is considered to be their fundamental difference from animal stem cells (Lohman, 2008; Verdeil et al., 2007).

As for the stem cells of invertebrates with asexual reproduction, traditionally named totipotent, it is usually not one stem cell, but some kind of a complex, an aggregate of the stem cells gives rise to the new organism or zooid in asexual reproduction (Blackstone & Jasker, 2003; Rinkevich et al., 2009). It was experimentally shown that one stem cell of a trypsinized cysticercus of the parasitic cestode *Taenia crassiceps* injected into the host organism may form a whole cysticercus in the host organism (Toledo et al., 1997), but it is a rare exception from the common rule. In rhizocephalan crustaceans, the endoparasitic organism develops from a few cells introduced into the host organism by the larva (Høeg & Lützen, 1995). The number of stem cells in the earliest bud of the blastozooid of the colonial rhizocephalans *Peltogasterella gracilis* and *Polyascus polygenea*, according to the author's data, is about 10–15 cells (Isaeva, 2010). The number of stem cells capable of producing the new individual in cnidarians, free living flat worms and colonial ascidians is estimated as lying between 100 and 300 (Rinkevich et al., 2009). A similar number of archaeocytes is required for producing a whole organism of the freshwater sponge *Ephydatia fluviatilis*, as experimentally determined by Nikitin (1977), who showed the necessity of a "critical mass" of cell aggregates for the development of a sponge organism. This "mass effect" is explained by the formation of sufficient concentrations of required metabolites in cell aggregates. The "critical mass" of cells is probably necessary also for the formation of an individual or a blastozooid in invertebrates. So, the ability of one cell or a small number of stem cells to develop into a whole organism is determined by an adequate environment.

To avoid confusion and misunderstanding, the term “pluripotent” is using here according to the recent reviews (Seydoux & Braun, 2006; Funayama et al., 2010; Strouji & Extavour, 2011). The problem of cell line having unlimited morphogenetic potential stems from A. Weismann’ “germ plasm” theory. August Weismann (1834–1914) was the first to discover and describe metazoan stem cells (*Stammzellen*) and primary germ cells (*UrKeimzellen*) in the course of his detailed study of colonial hydroids (Weismann, 1883; see also Frank et al., 2009). According to Weismann (1892, 1893), stem cells, retaining the “germ plasm” (*Keimplasma*, the nuclear hereditary substance containing the determinants of germ cells), are capable of differentiating into gametes, providing for the continuity of the immortal germ line, the germline way (*Keimbahn*) and the continuation of life in the sequence of generations. It was Weismann who determined that the germ cells of hydroids originate from the embryonic reserve of undifferentiated cells, and he did not associate the concept of germ cell line with the early isolation of this line (Weismann, 1892). Weismann has shown that the germ cells of hydroids differentiate not during embryonic development, but much later, in generations formed by budding (*Knospen-Generationen*: Weismann, 1883). The idea of early germ cell segregation and of the continuity of these cells in the sequence of generations was first of all formulated by Nussbaum (1880), who believed that interstitial cells of *Hydra* maintain the germline way. It was later shown that the interstitial cells of hydroids, stem cells continuously undergoing the mitotic cycle, produce both germ cells and some types of somatic cells (see Bode, 1996; Bosch, 2008; Frank et al., 2009). Thus, hydroids, with their late specification of germline cells, differentiating from the interstitial cells during the entire lifespan of the colony and producing also somatic derivatives, paradoxically became the main object of the studies that resulted in the emergence of the idea of early segregation of the totipotent germ cell line from somatic cells (Nussbaum, 1880), and to Weismann’s “germ plasm” theory (Weismann, 1883, 1892, 1893). Weismann supposed that germ cells preserve all the factors of inheritance, whereas each somatic cell loses, in the course of differentiation, part of the germ plasm and of the initial potential of the egg (Weissman, 1892, 1893); Weismann’s concept has been criticized in the light of modern biological data (see, e.g., Frank et al., 2009). But Weismann’s views were not as rigid as the views of some his followers: “Weismann was not a weismannist” (Winter, 2001, p. 518).

## 2.2 Self-renewal of stem cells

The term “self-renewal” denotes the ability of stem cells to reproduce mitotically during a long period, and in the case of the stem cells of adult organisms, during entire life span of the organism (Weissman et al., 2001; Lohman 2008; Rinkevich et al., 2009; Sköld et al., 2009). Particularly, pluripotent 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 & King, 2000; Hogan, 2000; Sköld et al., 2009).

For instance, sponge archaeocytes are self-renewing, mitotically active, telomerase-positive and bromodeoxyuridine incorporating cells (Müller, 2006; Funayama et al., 2010). 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 also capable of mitotic reproduction; so the stem cell system in hydroids includes interstitial, epidermal and gastrodermal stem cells continuously undergoing the mitotic cycle (Campbell, 1974; Thomas & Edwards, 1991; Bode, 1996). For the identification of stem cells capable of mitotic reproduction, bromodeoxyuridine, a thymidine analogue, was successfully used to reveal DNA synthesis in interstitial cells in hydra (Teragawa & Bode, 1990), and neoblasts in

flatworms (Gschwentner et al., 2001; Peter et al., 2001). Ethynyl deoxyuridine, another thymidine analogue, was employed for the same purpose in the ctenophore *Pleurobrachia pileus* (Alié et al., 2011). High activity of telomerase was shown in cells of the embryos, gonads, and early buds in colonial ascidian *Botryllus schlosseri* (Laird & Weissman, 2004).

PCNA (proliferating cell nuclear antigen) assay is used to reveal cells that do not cease to divide mitotically (Hall & Woods, 1990). The PCNA assay was also used to identify neoblasts in planarian; it has been shown that such a test is a reliable tool for neoblast identification in *Girardia japonica* (Orii et al., 2005). We used the immunochemical test PCNA to reveal proliferating cells in the colonial rhizocephalan *Peltogasterella gracilis* and the colonial hydroid *Obelia longissima*. Stem cells in budding stolons proved to be the only PCNA-positive cells in the *P. gracilis* interna while differentiated epithelial cells of the stolon and cells of the trophic system of the interna were PCNA-negative (Shukalyuk et al., 2005). Such test in *O. longissima* has revealed a more intense staining of interstitial and gonial cells, although reproducing epidermal and gastrodermal cells were also positive; differentiated somatic cells manifested poor or negative reaction (Isaeva et al., 2011).

Mammalian embryonic stem cells express telomerase, the protein associated with a pluripotent and immortal phenotype (Cogle et al 2003).

### 2.3 Gametogenic potentiality

The pluripotent stem cells ensure both sexual and asexual reproduction, being predecessors of the germ and all the somatic cells. The ability to differentiate into gametogenic and somatic cells was shown for archaeocytes and choanocytes in sponges (Simpson, 1984; Müller, 2006; Funayama, 2008; Funayama et al., 2010), interstitial cells in hydra and other cnidarians (Thomas & Edwards, 1991; Bode, 1996; Isaeva et al., 2011), turbellarian neoblasts (Shibata et al., 1999; Peter et al., 2001; Isaeva et al., 2005), stem cells of colonial rhizocephalans (Isaeva et al., 2004; Shukalyuk et al., 2005), ascidian stem cells (Pancer et al., 1995; Stoner & Weissman, 1996; Stoner et al., 1999; Weissman, 2000). So, pluripotent stem cells in invertebrates with asexual reproduction are potentially gametogenic cells.

Particularly, sponges have no permanent germline; archaeocytes and choanocytes are gametogenic cells (Tuzet, 1964; Blackstone & Jasker, 2003; Sköld et al., 2009). Sponge archaeocytes are considered to be the main cell source in sexual and asexual reproduction (Simpson, 1984; Müller, 2006; Funayama, 2008). Probably, the stem system of sponges includes two types of pluripotent stem cells: archaeocytes and choanocytes; both cell types are able to differentiate into germ and somatic cells; choanocytes can transform to archaeocytes, which later produce other cell types (Funayama, 2008; Funayama et al., 2010).

Cnidarian interstitial cells can produce germline cells (Campbell, 1974; Thomas & Edwards, 1991; Bode, 1996). The gonial cells and early oocytes developing from interstitial cells are distinct from them only by a greater size (Thomas & Edwards, 1991).

Gametogenic potentiality was most convincingly displayed using planarians (Baguña et al., 1989; Peter et al., 2001; Shibata et al., 1999). Neoblasts of asexually reproducing planarians and other Turbellaria are able to differentiate into germ and somatic cells of all types (Agata & Watanabe, 1999; Shibata et al., 1999; Peter et al., 2001; Orii et al., 2005). According to our data, neoblasts can become gonial cells: among individuals in asexual race of *Girardia tigrina* that reproduced exclusively by architomy during 40 years, one planaria laid cocoons after spontaneous sexualization; histological and ultrastructural study of this planaria demonstrated the presence of gonads, gonial cells, and oocytes (Isaeva et al., 2005).

Pluripotent stem cells of colonial rhizocephalans are also the predecessors of somatic and germ cells, so ensuring the reproductive strategy with alternation of asexual and sexual reproduction. In the colonial rhizocephalans *Polyascus polygenea* and *Peltogasterella gracilis* the stem cells migrated into the developing ovary becoming oogonial cells (Isaeva et al., 2004; Shukalyuk et al., 2005).

In colonial ascidians, germline as well as somatic cells differentiate from circulating hemoblasts (Pancer et al., 1995; Stoner & Weissman, 1996; Stoner et al., 1999). The differentiation of *Botryllus primigenus* hemoblasts into female germline cells was observed, and the primary germ cells are morphologically indistinguishable from hemoblasts (Sunanaga et al., 2006). Fusion of the vascular systems of colonies in ascidian *B. schlosseri* can lead to the replacement of germ and somatic cells in one colony with those from another one (Pancer et al., 1995; Stoner & Weissman, 1996; Stoner et al., 1999; Weissman, 2000; Laird et al., 2005). It remains unclear if the germline and somatic lineages are segregated or they descend from the same initial stem cell population in botryllids (Stoner et al., 1999).

Pluripotent embryonic stem cells of mammals have capability to differentiate *in vitro* into female and male germ cells (Hübner et al., 2003; Geijsen et al., 2003; Toyooka et al., 2003; Clark et al., 2004; Daley, 2007; Kerkis et al., 2007).

## 2.4 Morphological features of pluripotent stem cells

Pluripotent stem cells of asexually reproducing invertebrates and germline cells of all studied metazoan animals share common morphological and functional features: a high nuclear/cytoplasmic ratio, a large rounded nucleus with diffuse chromatin and a prominent nucleolus, thin rim of undifferentiated basophilic cytoplasm, including specific electron-dense cytoplasmic granules or nuage, and a set of specific regulatory molecules (Shukalyuk & Isaeva, 2005; Isaeva et al., 2008b, 2009, 2011; Extavour, 2008; Rinkevich et al., 2009; Isaeva, 2010; Strouji & Extavour, 2011; Shukalyuk et al., 2011). Germ cells can almost always be unambiguously distinguished from somatic cells by the same characteristic morphology (Extavour, 2008).

The morphological organization of pluripotent stem and gonial cells in the studied representatives of Porifera, Cnidaria, Platyhelminthes, Arthropoda, and Chordata shares common features typical for germline cells in other studied Metazoa (Isaeva et al., 2003, 2004, 2005, 2008b, 2009; Akhmadieva et al., 2007; Shukalyuk & Isaeva, 2005; Shukalyuk et al., 2005, 2007, 2011; Isaeva et al., 2011). For example, archaeocytes of the sponge *Oscarella malakhovi*, interstitial and gonial cells of the colonial hydroids *Obelia longissima* and *Ectopleura crocea* as well as neoblasts of the planarian *Girardia tigrina* have an ultrastructural morphology typical for stem and germ cells of all metazoan animals (Isaeva et al., 2005, 2011; Isaeva & Akhmadieva, 2011). Stem cells in the rhizocephalans *Polyascus polygenea* (Isaeva et al., 2004) and *Peltogasterella gracilis* (Shukalyuk et al., 2005) similarly demonstrate all morphological properties that are common to embryonic, pluripotent stem and germ cells of other metazoan animals.

### 2.4.1 Germinal granules (nuage)

#### Germline cells

The cells of the germ line can be identified and retraced during development of an organism owing to the availability of the “germ plasm” as cytoplasmic markers presented by granular or fibrillar material not surrounded by a membrane. “Germ plasm”, Weismann’s famous

term (Weismann, 1892, 1893) now is understood metaphorically. According to this modern understanding, the “germ plasm” contains electron-dense, RNA-enriched material structured as compact germinal granules or a more dispersed “nuage”, a specific ultrastructural marker of metazoan germline cells (see Matova & Cooley, 2001; Seydoux & Braun, 2006; Strome & Lehman, 2007). The germinal granules or nuage are considered key organelles of germline cells (Ikenishi, 1998; Amikura et al., 2001; Matova & Cooley, 2001; Chuma et al., 2006; Seydoux & Braun, 2006; Lim & Kai, 2007; Strome & Lehman, 2007).

The specific electron-dense material of germinal granules was denoted in the early XX century by the German terms *Keimbahnchromidien* and *Keimbahnplasma*, and this terminology was directly related to Weismann's; later the English terms *germ plasm*, *germ cell determinants*, *polar*, *perinuclear*, *chromatoid*, *germinal*, *germ granules (bodies)*, *dense bodies*, *ectosomes*, the French term *nuage*, and many others were introduced (see Mahowald, 1971, 2001; Beams & Kessel, 1974; Eddy, 1975; Ikenishi, 1998; Houston & King, 2000; Isaeva & Reunov, 2001; Matova & Cooley, 2001; Seydoux & Braun, 2006; Lim and Kai, 2007; Strome & Lehman, 2007; Isaeva et al., 2008b, 2009; Frank et al., 2009; Isaeva, 2010). Complex structures, including electron-dense material and mitochondria in vitellogenic oocytes, are called also the *Balbani body*, *vitelline (yolk) body*, *mitochondrial cloud*, *intermitochondrial cement*, *yolk nucleus*: “the chaos in the nomenclature” complicates the comparison of these structures in different species (Kloc et al., 2004).

The presence of germinal (perinuclear) granules is an evolutionary conserved feature 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 a few 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* (Mahowald, 1971). 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).

In the plant kingdom, oogonial cells of the brown alga *Undaria pinnatifida* include electron-dense bodies similar to the germinal granules of metazoan oogonial cells and oocytes; similar bodies were found in the cytoplasm of some other species of higher and lower plants (Alexandrova & Reunov, 2008), giving evidence of the common pattern of the morphofunctional organization of plant and animal reproductive cells. This similarity indicates a very conserved features of reproductive cells in Metazoa and Metaphyta.

### **Germlinal granules in pluripotent stem cells**

In some asexually reproducing invertebrates, stem cells capable to differentiate into germ and somatic cells can be also identified by the presence of specific electron-dense cytoplasmic structures, morphologically similar or identical to germinal granules or nuage in germline cells. The “germ plasm,” containing germinal granules or dispersed “nuage” material, becomes acceptable as a specific ultrastructural marker and a key organelle in pluripotent, potentially gametogenic stem cells of asexually reproducing invertebrates (Shibata et al., 1999; Mochizuki et al., 2001; Isaeva et al., 2008b, 2009, 2011; Frank et al., 2009; Strouji & Extavour, 2011), although the data concerning the structural and molecular organization of germ determinants in the cells of various metazoan taxa are rather fragmentary.

The germinal granules in stem cells of invertebrates, whose life cycle includes asexual reproduction, were earlier have been revealed in the interstitial cells of *Pelmatohydra robusta* (Noda, Kanai, 1977) and the planarian neoblasts (Hori, 1982; Rieger et al., 1991; Auladell et al., 1993; Agata & Watanabe, 1999; Shibata et al., 1999). Noda and Kanai (1977) shown that not only germline cells but also interstitial cells contain germ plasm with “dense bodies” associated with nuclear pores and mitochondria. The number and size of these dense bodies in *P. robusta* decreases as somatic cells (cnidoblasts) differentiate from interstitial cells and increases during early oogenesis (Noda & Kanai, 1977). In planarian, the germinal granules (chromatoid bodies) were observed both in the germline cells and in the neoblasts, where they likewise lie near the nuclear envelope in contact with the mitochondria (Hori, 1982; Auladell et al., 1993; Shibata et al., 1999). Chromatoid bodies disappear during neoblast differentiation into somatic cells, while in oogenic cells they are present throughout the life cycle (Hori, 1982; Auladell et al., 1993; Shibata et al., 1999). These observations led to a suggestion that chromatoid bodies have the function related to pluri/totipotency maintenance (Shibata et al., 1999).

Typical electron-dense germinal granules have not been previously described in the archaeocytes or any other cells of sponges. Dense fibrillar bodies were found in the oogonia and oocytes of different sponges (see Tuzet, 1964; Isaeva, Akhmadieva, 2011). In the cytoplasm of archaeocytes in the sponge *Oscarella malakhovi* we have found germinal granules of a typical morphology located near the nuclear envelope (Isaeva & Akhmadieva, 2011). The electron-dense germinal bodies revealed in interstitial cells of the colonial hydroids *Obelia longissima* and *Ectopleura crocea* (Akhmadieva et al., 2005; Isaeva et al., 2011) were similar in their ultrastructure to the “dense bodies” in interstitial and germ cells of *Pelmatohydra robusta* (Noda & Kanai, 1977) as well as in oocytes of *Hydra carnea* (Honegger et al., 1989) and other cnidarians (Thomas & Edwards, 1991). Such germinal granules are common for interstitial cells and oocytes of *O. longissima*. The germinal granules (usually termed chromatoid bodies) have been found near the nuclear envelope (often in contact with nuclear pores) surrounded by mitochondria in neoblasts and gonial cells of the planarian *Girardia tigrina* (Isaeva et al., 2005).

The cytoplasm of embryonic and stem cells in the studied rhizocephalans *Peltogasterella gracilis* and *Polyascus polygenea* contains germinal granules morphologically similar to those in germ cells. In particular, stem cells in rhizocephalan *P. gracilis* feature the presence of the germinal bodies with a typical ultrastructural morphology; all or most blastomeres of cleaving *Peltogasterella gracilis* embryos contain prominent germinal granules (Shukalyuk et al., 2005, 2007, 2011).

In the colonial ascidian *Botryllus tuberatus*, we observed in the cytoplasm of some stem cells in early buds small electron-dense bodies (Akhmadieva et al., 2007), which are similar to the nuage material often found in vertebrates.

So, pluripotent gametogenic stem cells in studied asexually reproducing sponges, cnidarians, turbellarians, arthropods, and chordates feature the presence of the germinal granules. So germinal granules (or more dispersed nuage material) can be used as a specific ultrastructural marker and a key organelle of pluripotent stem cells of asexually reproducing invertebrates (Shibata et al., 1999; Mochizuki et al., 2001; Isaeva et al., 2008b, 2009, 2011; Frank et al., 2009; Isaeva, 2010; Isaeva & Akhmadieva, 2011).

Electron-dense granular structures were observed also in embryonic stem cells of mouse (Shukalyuk et al., 2011). Thus, germinal granules were found not only in cells of the germ line but also in pluripotent stem cells of asexually reproducing invertebrates (sponges,

hydroids, turbellarians, colonial rhizocephalan crustaceans and ascidians) and pluripotent mESC *in vitro*.

In some somatic metazoan cells, processing bodies (P-bodies) have been found; their function is translation and they are considered as a structural and functional analog of the germinal granules (see Seydoux & Braun, 2006; Kotaja et al., 2006).

## 2.5 Molecular markers of stem cells

The ultrastructural and molecular organization of germinal granules of germline cells is evolutionarily conserved in all studied representatives of the animal kingdom from sponges to mammals (Ding & Lipshitz, 1993; Ikenishi, 1998; Houston & King, 2000; Matova & Cooley, 2001; Mochizuki et al., 2001; Extavour & Akam, 2003; Juliano et al., 2006; Seydoux & Braun, 2006; Strome & Lehman, 2007; Extavour, 2008; Ewen-Campen et al. 2010; Srouji & Extavour, 2011). It has been shown that some molecules localized in germinal granules are involved in specification of germline cells, and some genes encoding them are highly conserved evolutionary in all studied metazoans (Mahowald, 2001; Matova & Cooley, 2001; Mochizuki et al., 2001; Sato et al., 2001; Seydoux & Braun, 2006; Strome & Lehman, 2007). Germinal granules components include proteins, mRNAs, and noncoding RNAs; as far as is known, RNA-binding proteins are involved in mRNA localization, protection, and translation control (Extavour & Akam, 2003; Leatherman & Jongens, 2003; Chuma et al., 2006; Seydoux & Braun, 2006; Hayashi et al., 2007; Lim & Kai, 2007; Strome & Lehman, 2007; Ewen-Campen et al., 2010). The germinal granules are thought to function as a specific cytoplasmic regulatory center preventing the expression of somatic differentiation genes, maintaining the totipotency in germline cells, necessary for the conception of a new organism, preventing somatic gene expression and protecting the cells from somatic differentiation, that confirmed by data on the transcription "silence" of germline cells (Leatherman & Jongens, 2003; Chuma et al., 2006; Seydoux & Braun, 2006; Strome & Lehman, 2007; Cinalli et al., 2008; Extavour, 2008).

Germline cells can be distinguished from somatic cells by localization of mRNA or protein products of germ-cell-specific genes, notably the *vasa* and *nanos* gene family products (Extavour, 2008). Genes representing the core of the germline program, as *vasa*, *piwi*, *nanos* show striking evolutionary conservation (Extavour & Akam, 2003).

Several conserved molecules are expressed in both germ and pluripotent stem cells; these include Piwi family proteins, Tudor family proteins, and *PL10* gene products, *vasa* family members, and possibly *nanos* (Extavour, 2008; Ewen-Campen et al., 2010; Srouji & Extavour, 2011). Like germline cells, stem cells of asexually reproducing invertebrates are also characterized by the expression of protein products of genes related to *vasa*, *piwi*, *nanos*, and some other genes (reviews: Rinkevich et al., 2009; Sköld et al., 2009; Funayama et al., 2010). Nanos, a CCHC zinc finger RNA-binding protein expressed in germline stem cells in planarians (Sato et al., 2006), and in hydra (Mochizuki et al., 2000).

The piRNA-binding proteins of Argonaute subfamily, coding by *piwi*-related genes plays a central role in RNA silencing in a small RNA-mediated manner or via translational regulation; small regulatory RNAs include small interfering RNAs and Piwi-interacting RNAs mediating the epigenetic regulation of gene expression; these small RNAs can exert regulation at the transcriptional level, by affecting chromatin structure, or post-transcriptionally, by affecting mRNA stability or translation (Ambrose & Chen 2007). In metazoan germ cells, *piwi*-related genes express, presumably involving in germline and

stem cell maintenance; Piwi proteins as well as several microRNAs are highly evolutionary conserved within plant and animal kingdoms (Filipowicz et al., 2005; Ambrose & Chen 2007; Funayama et al., 2010; Shibata et al. 2010; Alié et al., 2011). Gametogenic pluripotent stem cells in sponges (Funayama et al., 2010), cnidarians (Seipel et al., 2004) and flatworms (Shibata et al., 2010) express *piwi* homologs.

So stem cells of invertebrates with asexual reproduction, as well as cells of the germ lineage, also display the expression of proteins related to Piwi, Nanos, and some others (reviews: Rinkevich et al., 2009; Sköld et al., 2009; Srouji & Extavour, 2011). In stem cells of the sponge *Ephydatia fluviatilis*, the activity of a gene related to *piwi*, which is expressed in germ and stem cells of other animals and plants and whose function is to maintain the totipotency (pluripotency) of these cells, was detected (Funayama, 2008; Funayama et al., 2010).

Mammalian embryonic stem cells express gene *Oct4* coding transcription factor associated with a pluripotent and immortal phenotype (Cogle et al., 2003), however, this gene unique to deuterostomes (see Srouji & Extavour, 2011). In undifferentiated mouse embryonic and induced pluripotent stem cells, which, along with invertebrate stem cells, are potentially gametogenic cells retaining pluripotency, expression of Miwi/Piwi, Nanog/Nanos and Oct4 was found (Shukalyuk et al., 2011). Thus, embryonic stem, germ and pluripotent stem cells of various metazoan animals share *piwi* gene expression.

### 2.5.1 Vasa and other members of DEAD-box family

The first identified component of the granules of germ plasm was the protein product of the *Drosophila vasa* gene (Hay et al., 1988), RNA helicase, belonging to the family of proteins containing conserved DEAD-box sequences (Luking et al., 1998; Raz, 2000; Extavour & Akam, 2003; Rosak & Linder, 2004). Proteins of the DEAD family are found in all eukaryotes (from yeast up to plants and animals) and are involved in splicing, editing, processing, nuclear-cytoplasmic traffic, initiation of translation, and degradation of RNA (Raz, 2000; Rosak & Linder, 2004). The Vasa family is thought to have evolved from the PL10 family of helicases, which share significant structural similarity with *vasa* gene products (Mochizuki et al., 2001). PL10 products are usually localized to both germ cells and pluripotent cell types (Extavour, 2008). Germinal granules of germline cells in various animals, from sponges to vertebrates, were found to contain a protein product (RNA-helicase) of the *vasa* gene or related genes, a key determinant and a universal marker of germline cells in metazoans, which is necessary for the formation and maintenance of the structural organization of germinal granules and, presumably, for the maintenance of pluri/totipotency of cells; in animals that reproduce only sexually, the expression of *vasa*-related genes is always exclusively confined to the germcell line during the entire course of development, from early embryo up to gametogenesis (Ding & Lipshitz, 1993; Ikenishi, 1998; Shibata et al., 1999; Castrillon et al., 2000; Raz, 2000; Matova & Cooley, 2001; Mochizuki et al., 2001; Extavour & Akam, 2003; Seydoux & Braun, 2006; Sunanaga et al., 2006; Strome & Lehman, 2007; Ewen-Campen et al. 2010). 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 specifics of the structural organization of the cytoplasm and of the functional activity of germline cells has resulted from evolutionarily conserved mechanisms common to all studied metazoan animals, and the presence of Vasa-like proteins in the germ plasm of different animals indicates the conservation of molecular mechanisms underlying the formation and maintenance of the germ plasm (Raz, 2000; Extavour & Akam, 2003; Juliano et al., 2006; Extavour, 2008; Ewen-Campen et al., 2010).

In the polyembryonic wasp *Copidosoma floridanum*, the secondary embryos develop either into normal larvae and then into fertile insects or into soldier larvae with the defense function. During development, at the stage of four blastomeres, one of them is Vasa-positive, and it gives rise to primary germline cells; embryos containing the Vasa-positive blastomere become fertile imagoes while the caste of soldiers without germline cells develops from the embryos depleted of the Vasa-positive blastomere, that confirms the involvement of Vasa 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 *Pelmatohydra robusta* (Mochizuki et al., 2001) and neoblasts of planarians (Shibata et al., 1999). Sequences of Vasa homologs have been found in the sponge *Ephydatia fluviatilis* (Mochizuki et al. 2000, 2001).

We have revealed the evolutionarily conserved sites of genes of the DEAD family, particularly *vasa* and *pl10* related genes, in DNA of the rhizocephalan crustaceans *Polyascus polygenea*, *Peltogasterella gracilis* and *Clistosaccus paguri* (Shukalyuk et al., 2007). Based on deduced sequencing of the protein products of these genes and on data from the genetic bank, a phylogenetic tree showing the close relationships of rhizocephalans to other arthropods was constructed. Selective expression of RNA of the *vasa*-related gene in stem and germline cells and its localization in the germinal granules of embryonic cells of *P. polygenea* were revealed. So selective expression of the *vasa*-related genes, the evolutionarily conserved markers and determinants of germline cells formerly revealed in germ cells of various metazoan animals and in stem cells of cnidarians and planarians, was also observed in embryonic, stem and germ cells of rhizocephalans (Shukalyuk et al., 2007). Thus, the expression of a gene related to *vasa* is specific not only to germline cells, but also to pluripotent gametogenic stem cells in asexually reproducing invertebrates, and the products of *vasa*-related genes involved in the determination of germ cells and in maintenance of cellular pluri/totipotency can be a useful molecular marker of pluripotent stem cells (Shibata et al., 1999; Mochizuki et al., 2001; Shukalyuk et al., 2007, 2009; Rinkevich et al., 2009; Sköld et al., 2009; Srouji & Extavour, 2011). The maintenance of the stem cell morphofunctional organization involves evolutionary conserved developmental mechanisms common for studied asexually reproducing multicellular animals.

Recently, Alié et al. (2011) found the expression of *piwi*, *vasa* and *PL10* in the male and female germlines and within pluri/multipotent stem cells in *Pleurobrachia pileus* (Ctenophora) without asexual reproduction. The authors suggest that *piwi*, *vasa* and *PL10* belong to a gene network ancestrally acting in two contexts: the germline and stem cells, whatever the nature of their progeny (Alié et al., 2011).

The data on *vasa*-, *PL10*- and *piwi*-related genes in pluripotent stem cells of invertebrates are presented in Table 1.

### 2.5.2 Mitochondrial components of germinal granules

The material of germinal granules (nuage) includes products of the nuclear genome; besides, there is evidence for the mitochondrial origin of some molecular components of germinal granules. The contact with mitochondria is a typical property of structured germinal granules in diverse multicellular animals (Isaeva & Reunov, 2001; Matova & Cooley, 2001; Carré et al., 2002). Ribosomal RNAs of mitochondrial origin and several other products of

	<i>vasa</i>	<i>PL10</i>	<i>piwi</i>
Porifera			Funayama et al., 2010
Cnidaria	Mochizuki et al., 2001; Rebscher et al., 2008	Mochizuki et al., 2001	Seipel et al., 2004
Ctenophora	Alié et al., 2011	Alié et al., 2011	Alié et al., 2011
Plathelminthes	Agata et al., 2006; Pfister et al., 2008	Shibata et al., 1999	Shibata et al. 2010
Annelida	Rebscher et al., 2007	Rebscher et al., 2007	Rebscher et al., 2007
Arthropoda (Rhizocephala)	Shukalyuk et al., 2007, 2011	Shukalyuk et al., 2007, 2011	
Echinodermata	Juliano & Wessel, 2009		Juliano et al., 2006
Chordata (Tunicata)	Rosner et al., 2009	Rosner et al., 2005	Brown et al., 2009

Table 1. Gene expression in pluripotent stem cells of invertebrates

the mitochondrial genome were revealed in the germinal granules of *Drosophila*, planarian and the frog *Xenopus* (Ding & Lipshitz, 1993; Kobayashi et al., 1998, 2005; Kashikawa et al., 1999; Amikura et al., 2001; Mahowald, 2001; Matova & Cooley, 2001; Leatherman & Jongens, 2003; Seydoux & Braun, 2006). 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). In *Xenopus* 16S rRNA can be found outside of mitochondria only in the germ plasm granules (Kobayashi et al., 1998). 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 rRNAs outside of the mitochondria in association with germinal granules has been generally accepted; it becomes apparent that mitochondrial rRNAs and other products of the mitochondrial genome are involved in the formation of germline cells in diverse multicellular animals (Ikenishi, 1998; Kloc et al., 2000; Mahowald, 2001; Amikura et al., 2001; Matova & Cooley, 2001; Leatherman & Jongens, 2003; Seydoux & Braun, 2006). It has been suggested that products of both the nuclear and mitochondrial genomes are essential for the structural organization and functioning of the germinal granules of germ plasm (Kobayashi et al., 1998, 2005; Ding & Lipshitz, 1993; Isaeva & Reunov, 2001; Isaeva et al., 2005, 2011).

The export of mitochondrial rRNA from mitochondria to the polar granules in *Drosophila* depends on the activity of nuclear genes *oskar*, *vasa*, and *tudor* (Amikura et al., 2001; Matova & Cooley, 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 (see Ding & Lipshitz, 1993). In addition to these proteins, the germ determinants include many more components encoded by the nuclear genome.

The export of the ribosomal RNAs from mitochondria to the germinal granules is no longer questioned, but the mechanism underlying a transport is considered unprecedented and enigmatic (Kashikawa et al., 1999; Ding & Lipshitz, 1993; Amikura et al., 2001). Our ultrastructural data indicating the disruption of the outer mitochondrial membrane and the

transformation of the mitochondrial matrix with inner membrane cristae into material of germinal granules in representatives of various animal taxa may clarify the mechanism of the export of mitochondrial components into germinal granules (Reunov et al., 2000; Reunov et al., 2004; Isaeva et al., 2005, 2011; Isaeva & Akhmadieva, 2011). This phenomenon enables us to suppose the participation of mitochondria in the biogenesis of the germinal granules (Isaeva & Reunov, 2001; Isaeva et al., 2005, 2011).

Destruction of the outer mitochondrial membrane and transformation of the mitochondrial matrix to the material of germinal granules or nuage have been revealed in the gonial cells of echinoderms and vertebrates (Reunov et al., 2000, 2004), planarian (Isaeva et al., 2005), sponge (Isaeva & Akhmadieva, 2011) and hydroids (Isaeva et al., 2011). The ultrastructural evidence of mitochondrial origin of the germinal granules (chromatoid bodies) in gonial cells and neoblasts of planarian *Girardia tigrina* has been obtained: the transformation of mitochondrial matrix with inner membrane cristae into the germinal bodies was observed (Isaeva et al., 2005). The mitochondrial derivatives devoid of the outer membrane but still containing flattened vesicles as remnants of inner membrane cristae is a usual picture observed in the germ granules or nuage of stem and gonial cells in the studied representatives of diverse taxa including the sponge *Oscarella malakhovi* and the hydroids *Obelia longissima* and *Ectopleura crocea* (Isaeva & Akhmadieva, 2011; Isaeva et al., 2011). We suggest that the structural frame of germinal granules derives from mitochondria and is filled with transcription products of the nuclear genome. The release of the mitochondrial matrix material in the germ plasm is the way to incorporate the mitochondrial derivatives into germinal granules, mediating the involvement of mitochondrial genome products in the biogenesis of the macromolecular complex of germinal determinants (Isaeva & Reunov, 2001; Isaeva et al., 2005, 2011). We propose also flows of molecular information connecting the nucleus, mitochondria, and germinal granules and involving in pluri/totipotency maintenance (Isaeva et al., 2005). The maintenance of the preexisted structural and functional organization of the germinal granules as cytoplasmic regulatory centers is likely controlled by ancient conserved mechanisms common for all multicellular animals (Isaeva & Reunov, 2001; Isaeva et al., 2005, 2011).

### 2.5.3 Alkaline phosphatase activity in stem cells

The histochemically detectable high level of alkaline phosphatase activity has become an empirical marker of mammalian primary germ and embryonic stem cells *in vivo* and *in vitro* (Chiquoine, 1954; Mintz, 1959; Talbot et al., 1993; Thompson et al., 1998; Lacham-Kaplan, 2004). High activity of alkaline phosphatase has been determined in the cultured embryonic stem cells, not only of mammals, but also of other vertebrates (for examples, Hong et al., 1998). Germ cells can be distinguished from somatic cells by high levels of alkaline phosphatase activity in vertebrate germline cells (Extavour, 2008).

No similar research has been carried out on invertebrates until quite recently. We applied cytochemical methods to show alkaline phosphatase activity for stem cell identification in the rhizocephalans *Peltogasterella gracilis* and *Polyascus polygenea* at the colonial parasitic stage of their life cycle to reveal the common feature of pluripotent stem cells of vertebrate and invertebrate animals. A high level of alkaline phosphatase activity, comparable to that of mouse embryonic stem cells *in vitro*, has been revealed in the cytoplasm of stem cells in the studied colonial rhizocephalans (Isaeva et al., 2003; Shukalyuk et al., 2005). The stem cells were identified due to their high alkaline phosphatase activity, in contrast with the

differentiated somatic cells of the endoparasitic interna characterized by a poor nonspecific staining of yellow color (Isaeva et al., 2003; Shukalyuk et al., 2005). In the blastomeres of dividing embryos of *P. polygenea* the alkaline phosphatase activity was confined to germinal granules located near the nucleus (Shukalyuk et al., 2005).

High alkaline phosphatase activity has also been recorded in interstitial and gonial cells of the colonial hydroid *O. longissima* (Isaeva et al., 2011). Histochemical assay for alkaline phosphatase revealed intense staining of some hemocytes (apparently, hemoblasts as stem cells) and cells of early buds in ascidian *Botryllus tuberatus* distinguished the stem cells from differentiated somatic cells (Akhmadieva et al., 2007).

Thus, the stem and gonial cells in rhizocephalans, hydroids and ascidians selectively express alkaline phosphatase activity. Specific brick red staining of stem cells in the studied representatives of colonial cnidarians, arthropods, and chordates was similar in color and intensity to that of cultured mouse embryonic stem cells used as “standard reference” (Isaeva et al., 2003; Shukalyuk et al., 2005). Our data is the evidence of the common functional characteristic of stem cells in such distant taxa as chordates, arthropods and cnidarians. We applied this classical histochemical method developed on the mammalian embryonic germ and stem cells to identify invertebrate stem cells, that reveals an opportunity for the application of this cytochemical reaction to the specific marking of stem cells of invertebrates in other taxonomic groups.

So classical reaction revealing the activity of alkaline phosphatase, earlier used for the identification of primary germ cells and embryonic stem cells in vertebrates, became applicable as a cytochemical marker of both gametogenic and pluri/totipotent stem cells of invertebrates (Isaeva et al., 2003; Laird et al., 2005; Shukalyuk et al., 2005; Akhmadieva et al., 2007; Rinkevich et al., 2009; Sköld et al., 2009). Among plants, a high alkaline phosphatase activity was found in the early gametangia of the brown alga *Undaria pinnatifida* (Alexandrova & Reunov, 2008).

## 2.6 Amoeboid cell motility of stem cells

Archaeocytes of sponges are characterized by amoeboid motility and active migration (Simpson, 1984; Müller, 2006; Funayama, 2008; Funayama et al., 2010). Archaeocytes are defined as large amoeboid cells actively migrating within the mesohyl (Funayama, 2008; Funayama et al., 2010). According to our data, migrating archaeocytes morphologically similar to those described previously in other sponge species participate in *Oscarella malakhovi* budding (Isaeva, Akhmadieva, 2011).

In hydra and other cnidarians, interstitial cells are capable of active migration (Campbell, 1974; Thomas & Edwards, 1991; Bode, 1996). Migration of numerous interstitial stem and oogonial cells inside the stolon and their participation in the formation of medusoid generation was also observed in *Obelia longissima* and *Ectopleura crocea* (Isaeva et al., 2011).

Turbellarian neoblasts can migrate to the injured surface and sites of gonad formation (Rieger et al., 1991; Auladell et al., 1993; Agata & Watanabe, 1999; Shibata et al., 1999); amoeboid neoblasts and gonial cells in planarian *Girardia tigrina* demonstrated the migratory possibility (Isaeva et al., 2005).

Undifferentiated rhizocephalan stem cells have been found inside each early stolon bud; similar cells migrate within the stolons in *Peltogasterella gracilis* and *Polyascus polygenea* (Isaeva et al., 2004; Shukalyuk et al., 2005).

The primary germline cells are known to emerge outside of the future gonad and later traverse through several developing somatic tissues on their journey to the emerging gonad

using both amoeboid motility and passive morphogenetic movements (Matova & Cooley, 2001; Kunwar & Lehmann, 2003; Travis, 2007; Cinalli et al., 2008).

Thus, pluripotent stem cells of asexually reproducing invertebrates are similar to primary germ cells in their ability to amoeboid movement and extensive migrations within the organism, directed to asexual reproduction sites, to the wound surface resulting from fission or damage, or to the gonads, respectively (Isaeva et al., 2009; Isaeva, 2010). In contrast, plant stem cells, with the rigid cellulose wall, are unable to migrate within the organism, and only passively moving together with the tissue, due to cell proliferation and expansion (Lohman, 2008).

### 2.7 Plasticity of stem cells in morphogenesis

Comparison of normal morphogenesis with its experimental changes helps us understand the plasticity of embryogenesis and blastogenesis. The plasticity of early animal embryogenesis is clearly shown in experiments with dissociated cells *in vitro* demonstrating the remarkable potential of cell self-organization (Isaeva et al., 2008a; Presnov et al., 2010). More than 100 years ago E. Wilson (1907) performed his famous experiments with dissociated sponge cells, reaggregated and developed into small sponges. Later Nikitin (1974) showed in experiments with dissociated cells of the sponge *Ephydatia fluviatilis* that homogeneous cell aggregates formed from stem cells (nucleolar amoebocytes, i.e. archaeocytes) are able to develop to a whole organism, while aggregates of other cell types inevitably died. Similar experimental studies were later carried out on sea urchin embryos: it was shown that reaggregates of dissociated embryonic cells *in vitro* formed “embryoids” (Giudice, 1962; Spiegel & Spiegel, 1986) which were able to develop into more or less normal larvae (Giudice, 1962) and, after metamorphosis, became fertile sea urchins (Hinegardner, 1975). Such experiments demonstrate the remarkable self-organization potential of embryonic stem cell *in vitro* (Isaeva et al., 2008a). Chimerical reaggregates of embryonic stem cells of sea urchins can form secondary blastula- or gastrula-like embryoids (Isaeva et al., 2008a; Presnov et al., 2010). This phenomenon is similar to the natural larval cloning by budding or fragmentation producing secondary larvae in starfish and other echinoderms (Jaekle, 1994; Vickery & McClintock, 2000; Rinkevich et al., 2009).

So changes in the initial conditions of morphogenesis *in vitro* lead to changed self-organization of an embryonic cell system; similar modifications of embryogenesis may occur also in the course of evolution under considerably changed conditions of early development, for example, in endoparasitism. The endoparasitic rhizocephalan interna with stem cells is “culturing” in the host organism and using host hemolymph as a nutritive medium; these extremely favorable conditions lead to stem cell proliferation and expansion of cells and tissues. In parasitoid insects with polyembryony, a system similar to cell culture is formed. Polyembryony, the development of a whole embryo from one of the early blastomeres, i.e. asexual reproduction at an early embryonic stage, is known at least in six animal phyla (Craig et al., 1997; Sköld et al., 2009). For instance, among insects, polyembryony has been described in some parasitoid members of Hymenoptera and Strepsiptera (Johannsen & Butt, 1941; Hagan, 1951). Polyembryony has been studied in detail in the parasitoid wasp *Copidosoma floridanum*: the zygote forms a morula, consisting of about 200 cells; repeatedly dividing mitotically active embryonic cells produce more than a thousand of secondary morulae, which form a polymorula, or polygerm (Donnell et al., 2004; Corley et al., 2005). Polyembryony is similar to budding (Perez, 1931; Ghiselin, 1987), and the latter is quite widespread in the animal kingdom as a way of asexual reproduction. In polyembryony, the

stage of cleavage, cell reproduction, becomes longer than in common embryonic development.

The embryonic stem cells of mammals *in vitro* are similar to the pluripotent stem cells of rhizocephalans or other asexually reproducing animals. Stem cells in free-living asexually reproducing invertebrates use the own parental organism as a nutritive medium. Germ and pluripotent stem cells are “privileged”, “predatory” cells inclined to “parasitism” as has been displayed for colonial ascidians (Buss, 1999; Pancer et al., 1995; Rinkevich, 2009); these cells can survive starvation through “cannibalism” (Kerszberg & Wolpert, 1998).

Sexual reproduction and early stages of embryogenesis are relatively conservative in all the animal kingdom due to the monophyly of metazoans (Sköld et al., 2009). Since asexual reproduction emerged in the course of the evolution of different metazoan lineages repeatedly and independently, asexual reproduction is more variable and less conservative than embryogenesis. The stage of cleavage is missing in blastogenesis, and stem cells can be likened to the embryonic cells of the morula stage. The integration of blastogenesis in the process of early embryogenesis in animals with polyembryony disrupts the conservatism of embryonic development (Isaeva, 2010). Polyembryony and the breaking of the conservatism of embryogenesis are known also in plants (Batygina, 2010).

The data on the asexual reproduction in some arthropods and chordates contradicts the dogma that asexual reproduction is common exclusively among the lower animals. In particular, the statement that vertebrates are incapable of natural cloning (Blackstone & Jasker, 2003) is disproved by known facts about facultative polyembryony in mammals, which has become obligate in some armadillo species, e.g. in *Dasypus novemcinctus* (Loughry et al., 1998).

The self-renewing pool of totipotent stem cells in colonial invertebrates provides the cellular basis for realization of the reproductive strategy including both asexual and sexual reproduction. The principal difference between the reproductive strategy that includes asexual reproduction and the strategy with exclusively sexual reproduction concerns the maintenance of the pluri/totipotent stem cell lineage with gametogenic potential during the entire life span of an asexually reproducing organism; a self-renewing reserve of pluripotent stem cells is the cellular source ensuring the reproductive strategy that includes sexual and asexual reproduction.

The problem of cells dedifferentiation in asexual reproduction and regeneration is less obvious, and the solution of this problem requires special markers. The notion of the high plasticity of the development and fate of cells in colonial animals (Frank et al., 2009; Rinkevich et al., 2009; Sköld et al., 2009), similar to that found in plants (Skold et al., 2009), appears sufficiently justified. In plants, however, differentiated cells retain the ability to dedifferentiate and become totipotent stem cells (Lohman, 2008; Batygina, 2010); animal cells at the stage of terminal differentiation usually have no such ability.

## **2.8 Evolutionary transition from preformation to epigenesis in colonial Rhizocephala**

Extavour (2008) considered the transition from epigenesis to preformation as the repeated evolutionary event, but she thinks that examples of epigenesis in phyla where preformation is plesiomorphic never observed. However, the blastogenesis in colonial species of rhizocephalan crustaceans (Arthropoda: Crustacea: Cirripedia: Rhizocephala) involves a deep reorganization of development; we observe evolutionary secondary transition from preformation to epigenesis. We found germinal bodies in all or most blastomeres of cleaving embryos of *Polyascus polygenea* at stage of 16-32 blastomeres. In each germinal body selective expression of mRNA transcript of *vasa*-like gene and also high selective activity of alkaline

phosphatase were revealed (Shukalyuk et al., 2005, 2011). These data indicates the evolutionary secondary rearrangement of the developmental mode and transition from ancestral preformation with mosaic cleavage and early segregation of the germ line (that is plesiomorphic feature in Crustacea and all Arthropoda) to epigenesis with equipotential blastomeres containing germinal granules and asexual reproduction resulting in colonial organization (Isaeva, 2010). Thus, the evolutionary transition from preformation to epigenesis is possible as well as more frequent evolutionary transition from epigenesis to preformation. In Rhizocephala, the radical transformation of the ancestral reproductive strategy involved all the levels of organization, from molecular and subcellular to species-specific (Kasyanov, 2001). The cellular basis for the reproductive strategy of Rhizocephala, including both sexual and asexual reproduction, is self-renewing pool of pluri/totipotent stem cells (Isaeva et al., 2008b, 2009).

In recent reviews (Blackstone & Jasker, 2003; Sköld et al., 2009) and in modern textbooks, crustaceans as well as all other arthropods and the entire Ecdysozoa clade, are considered as a colonial and a clonal, though in some rhizocephalan crustaceans the colonial organization as result of asexual cloning has already been described (Høeg & Lützen, 1995; Isaeva et al., 2003, 2004, 2008; Glenner et al., 2003; Shukalyuk et al., 2005). We have shown budding of a stolon filled with stem cells in colonial rhizocephalans *P. polygenea* and *P. gracilis* (Isaeva et al., 2003, 2004, Shukalyuk et al., 2005, 2007). Stem cells in rhizocephalans demonstrate all morphological properties shared by stem cells in other studied animals with asexual reproduction. The colonial nature of Rhizocephala has probably been denied because their colonial interna was not clearly visualized until quite recently. We have visualized the process of asexual reproduction by budding with maintaining colonial unity and connections between the developing blastozooids and common stolon in *P. polygenea* and *P. gracilis*, leaving no doubt about the colonial organization of these crustaceans at the endoparasitic stage of their life cycle (Isaeva & Shukalyuk, 2007; Isaeva et al., 2008b; Isaeva et al., 2004; Shukalyuk et al., 2005, 2007).

### 3. Conclusion

On the morphological and gene expression levels, germ cells and stem cells are very similar (Shukalyuk & Isaeva, 2005; Extavour, 2008; Isaeva et al., 2008b, 2009, 2011; Rinkevich et al., 2009; Sköld et al., 2009; Isaeva, 2010; Srouji & Extavour, 2011). Pluri/totipotent gametogenic stem cells are similar to germ and embryonic stem cells; evidence of the evolutionary conserved morphological and functional characteristics of pluripotent stem cells typical also to cells of the germ line have been obtained in representatives of such various metazoan phyla as Porifera, Cnidaria, Plathelminthes, Arthropoda and Chordata (Isaeva et al., 2003, 2004, 2005, 2008b, 2009, 2011; Shukalyuk et al., 2005, 2007, 2011; Akhmadieva et al., 2007; Isaeva & Akhmadieva, 2011).

The data supported our hypothesis that pluripotent, potentially gametogenic stem cells display evolutionarily conserved features of the morphological and functional organization typical for cells of the germ line and embryonic stem cells. In asexually reproducing invertebrates, from sponges and hydroids to some arthropods and chordates, stem cells share with cells of early embryos evolutionary conserved features presumably involved in maintenance of pluri/totipotency, including the gametogenic program. Such invertebrate cells capable of both gametogenesis and asexual reproduction (blastogenesis) are similar in their potential to mammalian embryonic stem cells. We propose that evolutionary and

ontogenetically related cells of early embryos and pluripotent stem cells belong to populations of cells that retain a wide or unlimited morphogenetic potential.

Our results along with literature data allow suggest the existence of evolutionary conservative, common for all studied metazoan representatives, from sponges to chordates, cellular, sub-cellular and molecular bases of pluripotency and “stemness” of stem and germ cells.

Many authors called pluripotent stem cells of animals with asexual reproduction *somatic* cells (Blackstone & Jasker, 2003; Extavour & Akam, 2003; Extavour, 2008; Rinkevich, 2009; Sköld et al., 2009; Funayama et al., 2010). The term *somatic embryogenesis* (Buss, 1987; Blackstone & Jasker, 2003) clearly shows that stem cells providing for asexual reproduction are considered as somatic cells. However, pluri/totipotent stem cells in asexually reproducing animals, as well as primary germ cells in sexual reproduction, are not belonging to any germ layer, tissue, or population of specialized somatic cells (Isaeva et al., 2008b, 2009; Isaeva, 2010). The population of these pluripotent stem cells is a diaspora of amoeboid cells, dispersed in the organism; these stem cells do not display contact inhibition of cell reproduction and movement. The morphological and functional organization of the stem cells capable for both gametogenesis (and subsequent embryogenesis) and asexual reproduction (blastogenesis) and germ-line cells shares common properties. Our notion of the evolutionary and ontogenetic similarity between the stem cells of asexually reproducing animals, cells of the germ line and embryonic stem cells (Isaeva et al., 2008b, 2009) brings us back to the concept of Weismann (1883), who wrote about the indistinguishability of gametogenic cells and undifferentiated cells that preserve the “germ plasm.” It was later proposed that pluripotent gametogenic stem cells and getmline cells could be evolutionarily and ontogenetically related (Weissman, 2000; Extavour & Akam, 2003; Travis, 2007; Extavour, 2008; Strouji & Extavour, 2011). Primary germ cells and pluri/totipotent stem cells share many morphological characters and rely on the activity of related genes (Extavour & Akam, 2003; Hayashi et al., 2007; Travis, 2007; Sköld et al., 2009; Srouji & Extavour, 2011). Pluripotent stem cells of animals with asexual reproduction were termed “primary stem cells” (Sköld et al., 2009); such pluripotent stem cells are predecessors of primary germ cells (Blackstone & Jasker 2003; Sköld et al., 2009). Evolutionary conserved mechanism ensures germline specification remaining cell pluripotency (Strouji & Extavour, 2011). Thus, the pluripotent stem cells are not identical to somatic cells. Besides, morphogenesis in animal asexual reproduction does not completely recapitulate embryogenesis. So, the term *somatic embryogenesis* (Buss, 1987; Blackstone & Jasker, 2003) is not completely correct, the term *blastogenesis* seems preferable (Berrill, 1961).

Stem cells of animals with asexual reproduction, as well as cells of the germ lineage, probably originate in the early embryogenesis either from the early totipotent blastomeres or from their derivatives that retain pluri/totipotency. The author believes that the evolutionarily and ontogenetically related cells of early embryos, primary stem and primary germ cells belong to cell populations capable of realizing the developmental program, including gametogenesis (and, potentially, subsequent embryogenesis) and blastogenesis (Isaeva et al., 2008b, 2009; Isaeva, 2010).

Thus, published and original data indicate evolutionary conservation and similarity of the studied morphofunctional properties of stem cells in metazoans with asexual reproduction (from sponges and cnidarians to chordates), germline and embryonic stem cells. In invertebrates with asexual reproduction, stem cells can differentiate into both germline and somatic cells; these pluri/totipotent stem cells represent a source of cells for the life strategy realization including sexual and asexual reproduction. Further research on the stem cells of various metazoan animals may reveal the evolutionary conserved basis of cellular totipotency and potential immortality.

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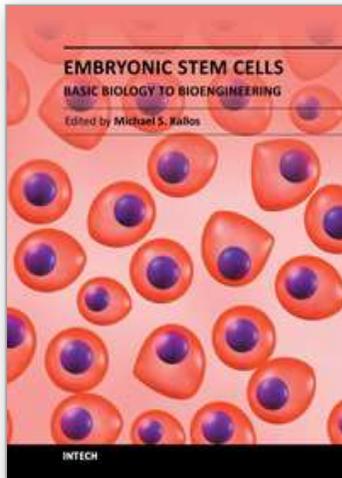
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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides a snapshot of some of the research occurring across a wide range of areas related to embryonic stem cells, including new methods, tools and technologies; new understandings about the molecular biology and pluripotency of these cells; as well as new uses for and sources of embryonic stem cells. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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