ISSN 1062-3604, Russian Journal of Developmental Biology, 2014, Vol. 45, No. 4, pp. 205–223. © Pleiades Publishing, Inc., 2014.
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REVIEWS

Sponge Cell Reaggregation: Mechanisms and Dynamics of the Process

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Received August 6, 2013; in final form, February 20, 2014

Abstract—Sponges (Porifera) are lower metazoans whose organization is characterized by a high plasticity of anatomical and cellular structures. One of the manifestations of this plasticity is the ability of sponge cells to reaggregate after dissociation of tissues. This review brings together the available data on the reaggregation of sponge cells that have been obtained to date since the beginning of the 20th century. It considers the behavior of dissociated cells in suspension, the mechanisms and factors involved in reaggregation, and the rate and stages of this process in different representatives of this phylum. In addition, this review provides information about the histological structure of multicellular aggregates formed during reaggregation of cells and the regenerative morphogenetic processes leading to the formation of normal sponges from these multicellular aggregates.

Keywords: sponges, reaggregation of cells, primmorphs, regenerative morphogenesis

DOI: 10.1134/S1062360414040067

INTRODUCTION

The origin and early evolution of metazoans is one of the most important problems in modern biology. Multicellularity could not occur without the appearance of coordinating mechanisms of cell division, differentiation, and programmed death, as well as without cell–cell adhesion and recognition. The evolution of these mechanisms ensured more and more stable integration of individual cells in united coordinated structures, which ultimately led to the transition from protists to metazoans through colonial forms. To date, ample data on the behavior of cells and the mechanisms of their integration and communication in intact tissues, during embryonic development, and during regenerative morphogenetic processes in a large number of invertebrates and vertebrates have been obtained. The analysis of these data made it possible to identify the common patterns of tissue functioning in metazoans and the putative processes that occurred in the early stages of their evolution (Tyler, 2003; Srivastava et al., 2010; Adamska et al., 2011). However, many problems in this field remain unsolved. A promising approach to ensure further progress in solving these problems is to study in detail the mechanisms of integration and communication of cells in tissues of lower metazoans, in particular, representatives of phylum Porifera (sponges).

Sponges are aquatic sedentary metazoans with a filtration type of nutrition and respiration. Adult sponges have a very primitive organization: they do not have any organs or digestive, nervous, and muscular systems. The most characteristic feature of organization of sponges is their water-current system—a network of canals running throughout the body and connecting choanocyte cameras lined with choanocytes, flagellated collar cells. Water enters the water-current system through ostia (numerous small holes on the sponge surface) and is ejected through oscula (one or several large holes) (Fig. 1).

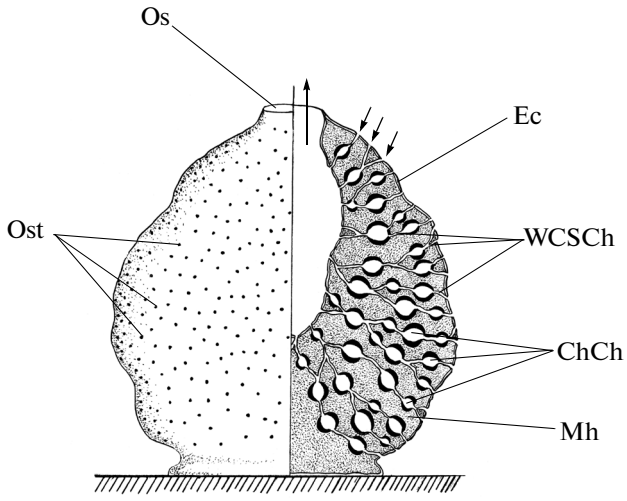

Fig. 1. Scheme of organization of an adult sponge (by: Lavrov and Kosevich, 2013). Designations: (ChCh) choanocyte chambers, (WCSCh) water-current system canals, (Mh) mesohyl, (Os) osculum, (Ost) ostia, (Ec) pinacoderm. Arrows on the scheme show the direction of water currents through the water-current system.

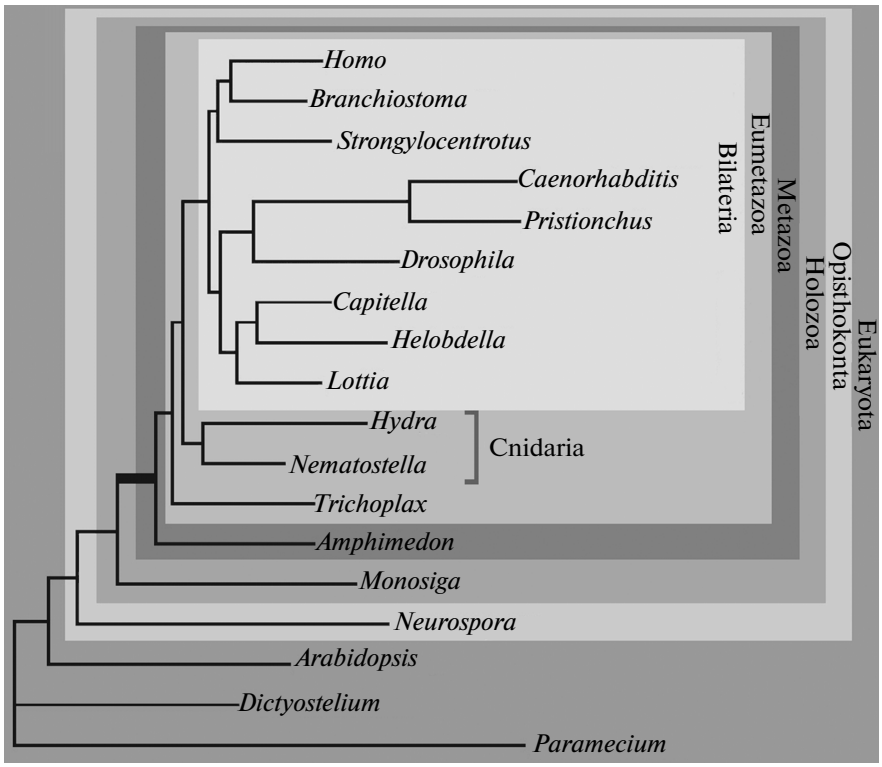

Fig. 2. Phylogenetic tree of multicellular animals built by the Biesian method on the basis of 229 nuclear protein-coding genes. All clades have statistical support in the form of posterior probability equal to one (by: Srivastava et al., 2010).

The water-current system ensures pumping huge amounts of water through the sponge body, from which it receives food and oxygen required for life. Due to the constant beating of flagella, choanocytes create a water current running through the animal body. Choanocytes also provide the sponge with food (food particles are captured from the water primarily by these cells). The canals of the water-current system and the outer surface of the sponge body are covered with a layer of flattened or T-shape cells—pinacocytes.

The entire volume of the body between the canals, chambers of the water-current system, and the external surface of the sponge is occupied by the so-called mesohyl. Although the thickness of the mesohyl varies in different groups of sponges, in all representatives of the phylum Porifera, it includes a large number of mobile cell types differing in structure and functions (archaeocytes, sclerocytes, collencytes, etc.) and dispersed in a watered extracellular matrix (basic substance of mesohyl) (Ereskovsky, 2005). In addition, mesohyl contains the animal's skeleton, which is composed of organic (spongin bands) and inorganic (calcareous or siliceous spicules) elements (Fig. 1).

For a long time, sponges were considered as an independent branch of animals that evolved independently of Metazoa. However, many researchers characterized sponges as animals that are between protists and true metazoans (Hadzi, 1963; cited by Ereskovsky, 2005). This standpoint was based on the structural features of the body of sponges and the similarity of choanocytes with unicellular organisms or colonial protists choanoflagellates (Adamska et al., 2011).

With the development of molecular biological techniques, it became obvious that sponges and true multicellular animals (Eumetazoa) form a single monophyletic group (Fig. 2). Recent papers on this subject indicate not only the monophyly of sponges and Eumetazoa but also the basal position of sponges in the metazoan clade (Srivastava et al., 2008, 2010; Pick et al., 2010). Moreover, there is reason to assume that Porifera is currently the only existing type of animal that is close to the hypothetical ancestor of metazoans (Müller, 2001). In this regard, it is impossible to discuss the issues of emergence and early evolution of metazoans without taking into consideration the sponges.

The monophyly of sponges and Eumetazoa suggests that the molecular mechanisms that ensure the functioning of tissues, cell communication and integration, as well as morphogenesis in sponges, are similar to those in higher metazoans. To date, it was found that the genome of sponges contains genes encoding many classes of transcription factors (ANTP, PRD, POU, LIM, SIX, Sox, nuclear receptor (NR), Fox, T-box, Mef2, Ets, and IRO) and components of the main signaling pathways (Wnt, TGF-β, Notch, and Hedgehog), which are typical for representatives of

higher metazoans (Larroux et al., 2006; Srivastava et al., 2010; Ereskovsky et al., 2013).

However, some features in the organization of sponges are absolutely uncharacteristic of other metazoans. A unique feature of the organization of adult sponges is the mobility and plasticity of their anatomical and cellular structures. Unlike other metazoans at postembryonic stages, in which active cell movements and differentiation are associated primarily with the formation of structural and functional components of the organism or with the regenerative morphogenetic processes, all cells in the sponge body are in constant movement and undergo transdifferentiation. This leads to a continuous reorganization of the main anatomical structures of the animal's body—the water-current system and the skeleton. The high lability of all structures in the sponge's body allows them to quickly and adequately respond to changing hydrodynamic conditions of the environment (Bond, 1992; Ereskovsky, 2005).

One of the manifestations of this plasticity is the ability of sponge cells to reaggregate after dissociation of animal's tissues induced by chemical or mechanical methods. The process of reaggregation of sponge cells was first described by Wilson in 1907 (Wilson, 1907) and has since become the subject of studies by many authors. Both the morphological aspect of the process and the issues related to cell–cell interactions, dedifferentiation, and transdifferentiation of various cell types have been studied (Efremova, 1969; Volkova and Zolotareva, 1981). Reaggregation involves the formation of multicell aggregates of various structures and, in some cases, complete regeneration of sponge organization (Wilson, 1907; Huxley, 1912; Galtsoff, 1925b; Korotkova, 1972). A special stage of this process is the formation of the so-called primmorphs. Primmorphs represent three-dimensional cell aggregates of a spherical shape. They are characterized by the presence of a continuous pinacoderm separating the inner mass of unspecialized cells from the environment. Primmorphs are the stage of completion of cell reaggregation. After their final formation, the processes of sponge regeneration and growth can begin under certain conditions (Custudio et al., 1998; Müller et al., 1999). Long-term cultures of primmorphs are currently regarded as a potential system for obtaining biologically active substances from sponges (Osinga, 1999; Müller et al., 2000; Pomponi, 2006).

The purpose of this review was to summarize the currently available data on various aspects of sponge cell reaggregation. It analyzes the behavior of cells during reaggregation, the dynamics of formation and the structure of cell aggregates, the mechanisms and factors that determine the reaggregation of cells, and the processes leading to the restoration of the intact organization of the animal.

BEHAVIOR OF CELLS AT THE EARLY STAGES OF REAGGREGATION

Sponge cell suspensions are usually obtained using two basic tissue dissociation methods—chemical and mechanical. In the case of mechanical dissociation, animal's tissues are rubbed through small-mesh grinding gauze, which leads to their mechanical separation to single cells and small cell clumps (Wilson, 1907; Efremova, 1969). In the case of chemical dissociation, sponge tissues are placed in calcium- and magnesium-free water containing a chelating agent (EDTA), which leads to the disruption of cell–cell contacts and separation of tissues to individual cells (Humphreys, 1963; Custudio et al., 1998; Müller et al., 1999). Concentrated cell suspensions are placed in Petri dishes, thus obtaining temporary cell cultures in which reaggregation will be observed.

Reaggregation begins in the cell suspension immediately after its preparation (if the suspension was obtained by the chemical method, reaggregation begins only after the transfer of cells to a medium containing Ca^{2+} and Mg^{2+} ions. In suspension, isolated cells rapidly acquire a spherical shape. An exception to this trend is choanocytes, which retain the flagellum and the collar of microvilli for some time. Cells begin to form pseudopodia whose number, size, and shape significantly vary (Galtsoff, 1923; Efremova and Drozdov, 1970; Efremova, 1972; Korotkova, 1972; Volkova and Zolotareva, 1981). In some cases, it was shown that pseudopodia are formed by only certain types of cells (amoebocytes), whereas in other types of cells (choanocytes and/or pinacocytes) this ability is either absent (Korotkova, 1972) or manifested to a lesser degree (Efremova and Drozdov, 1970; Efremova, 1972; Korotkova, 1997).

After settling from suspension on a substrate and formation of pseudopodia, cells of most sponge species acquire amoeboid motility. Wilson, who worked with marine sponges *Clathria prolifera* (Ellis and Solander, 1786) from class Demospongiae, noted that the amoeboid motility is characteristic of all cells of this species, although he did not study in detail the behavior of cells in suspension (Wilson, 1907). More detailed observations of the behavior of *C. prolifera* cells in suspension were performed by Galtsoff (1923, 1925a). Galtsoff distinguished in suspension three types of cells—archaeocytes, coating cells (probably pinacocytes), and choanocytes. According to his observations, amoeboid motility was characteristic of only archaeocytes and coating cells (archaeocytes moved more actively), whereas choanocytes could move only for some time due to the beats of the flagellum. Similar data on the behavior of cells in suspension were obtained for the freshwater sponges *Ephydatia fluviatilis* (Linnaeus, 1759) and *Spongilla lacustris* Linnaeus, 1759 (Yazykov, 1965; Efremova and Drozdov, 1970; Efremova, 1972). Evidence for the ability of only certain types of cells (amoebocytes) to

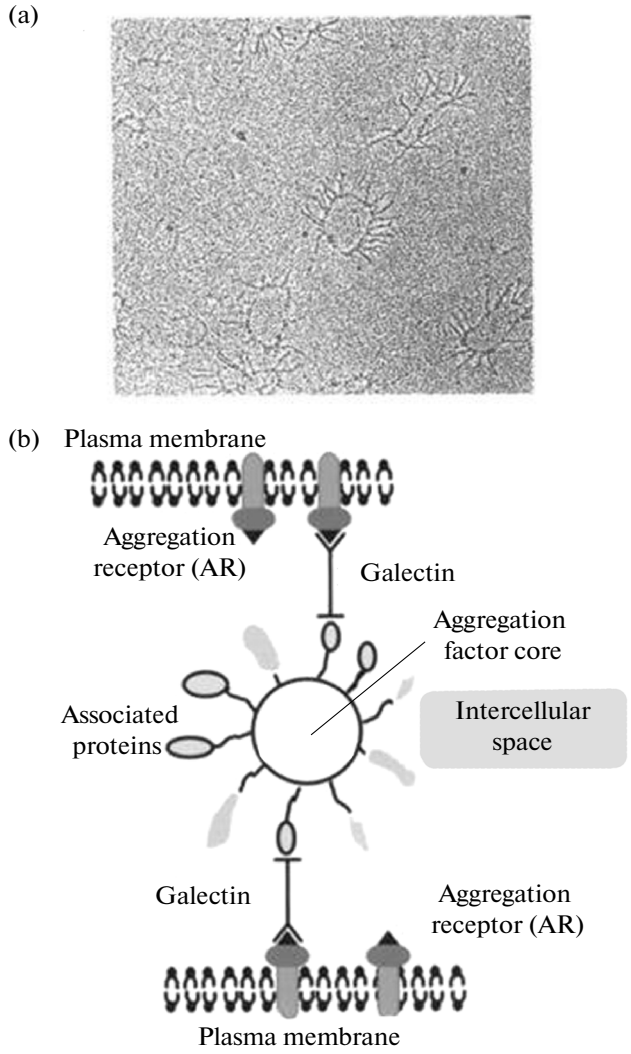

Fig. 3. Aggregation factor (AF) of sponge cells (by: Müller, W.E.G. and Müller, I.M., 2003). (a) Electron micrograph of the aggregation factor of *Geodia cydonium* (Jameson, 1811). The core of the complex and 25 proteins associated with it can be seen (TEM, magnification ×70000). (b) Scheme of interaction of sponge cells through the aggregation factor.

perform amoeboid movements was also obtained for two representatives of class Calcarea—*Leucosolenia complicata* (Montagu, 1818) and *Sycon lingua* (Haeckel, 1870) (Korotkova, 1972; Giano et al., 1985).

Observations of cell migration in *C. prolifera* (Galtsoff, 1923) and *Clathrina* sp. (Giano et al., 1985) showed that cells migrate in random directions; i.e., cells do not perform targeted movements to each other or towards a large group of cells. During migration, cells often change the direction and speed of their movement. When cells encounter each other in the course of migration, they aggregate and form small groups, which also retain motility. Apparently, in these sponge species, cells reaggregate mainly due to random encounters of motile cells or groups of cells (Wilson, 1907; Galtosoff, 1923, 1925a; Giano and Burlando, 1990).

Another mechanism of reaggregation was described for a number of species of marine Demospongiae (*Halisarca dujardini* (Johnston, 1842), *Haliclona aquaeductus* (Schmidt, 1862), and *Halichondria panicea* (Pallas, 1766)). In suspension, cells of these sponge species become rounded and form pseudopodia but do not exhibit amoeboid activity. Cells use pseudopodia to examine the space around them. Cells approach one another only when pseudopodia come into contact (Volkova and Zolotareva, 1981; Lavrov and Kosevich, 2013).

Nevertheless, there are data on the ability of *H. panicea* cells to active amoeboid movement in suspension (Yazykov, 1965). Thorough observations of *E. fluviatilis* cells in suspension revealed a "mixed" pattern of their behavior: the majority of cells form pseudopodia for active movement over substrate, but some cells use pseudopodia for only "searching" movements around them (Efremova and Drozdov, 1970; Efremova, 1972). Probably, the behavioral pattern of dissociated sponge cells is not a species-specific phenomenon and largely depends on the experimental conditions and the physiological state of the sponge from which the suspension was obtained.

MOLECULAR MECHANISM OF CELL REAGGREGATION

At the molecular level, sponge cells reaggregate with the involvement of an extracellular macromolecular complex—aggregation factor. Electron and atomic force microscopy studies showed that the aggregation factors of *C. prolifera* and *Geodia cydonium* (Jameson, 1811) are rosette-shaped structures, in the center of which the core of the complex (a circular glycosylated protein molecule 100–130 nm in diameter) is located, from which 16–25 linear protein molecules radiate; the latter are connected with the core through covalent and noncovalent bonds (Fernández-Busquets and Burger, 1999; Müller, W.E.G. and Müller, I.M., 2003) (Fig. 3a). The aggregation factors of *Halichondria bowerbanki* (Burton, 1930), *Suberites aurantiacus* (Duchassaing and Michelotti, 1864), and *Haliclona oculata* (Pallas, 1766) have similar size and structure; however, the core of the complex is represented by a linear molecule (Fernández-Busquets and Burger, 1999). The aggregation factor passes into solution after dissociation of intact sponge tissues. During reaggregation of cells, it functions as a peculiar bridge connecting adjacent cells and interacting with the aggregation receptor, a transmembrane protein, which leads to the association of these cells. Experiments with suspensions of *C. prolifera* and *H. oculata* cells showed that the concentration of free aggregation factor in the medium decreases in the course of reaggregation, because it binds to cell surfaces (Moscona,

1968). The reaggregation of cells mediated by the aggregation factor is a two-step process including Ca^{2+}-dependent and Ca^{2+}-independent steps. Therefore, the presence of Ca^{2+} and Mg^{2+} ions and the polypeptide galectin in the medium is required for effective functioning of the aggregation factor (Spiegel, 1954; Humphreys, 1963; Korotkova, 1997; Popescu and Misevic, 1997; Fernández-Busquets and Burger, 1999; Müller, W.E.G. and Müller, I.M., 2003; Ereskovsky, 2007) (Fig. 3b).

The initial concentration of the aggregation factor in the medium affects the degree of reaggregation of sponge cells: an increase in its concentrations leads to the formation of larger aggregates, whereas a decrease in its concentration leads to a reduction in the size of aggregates or the absence of aggregation (Humphreys, 1963; Moscona, 1968; Leith, 1979). For aggregation, sponge cells should not be necessarily metabolically active. In the presence of the aggregation factor, the reaggregation of cells preliminarily fixed in glutaric aldehyde or formalin (Moscona, 1968; Leith, 1979) or the reaggregation of cells at low temperatures, when the metabolism of cells is blocked, can be observed (Humphreys, 1970; Popecsu and Misevic, 1997; Fernández-Busquets and Burger, 1999). However, if the aggregation factor itself is absent or blocked (with immune serum or polyclonal antibodies), reaggregation of cells does not occur (Spiegel, 1954; Humphreys, 1963; Popecsu and Misevic, 1997; Fernández-Busquets and Burger, 1999).

SPECIES AND INDIVIDUAL SPECIFICITY OF CELL REAGGREGATION

It is known that, during reaggregation, cells of some sponge species can undergo *species-specific aggregation*. In this case, the during mixing of cell suspensions obtained from two different sponge species, reaggregation only between the cells and cell aggregates of the same species is observed. Cells of different species do not reaggregate even if they are artificially brought close to one another by centrifugation (Wilson, 1910; Galtsoff, 1923, 1925a; Curtis, 1962; Humphreys, 1963, 1970; Fernández-Busquets and Burger, 1999).

Experiments of Humphreys (1963, 1970) with a mixed suspension of *C. prolifera* and *H. oculata* cells showed that a highly species-specific reaggregation is characteristic of these species and that mixed aggregates are never formed in cultures. When aggregation factors of both species are removed from the mixed cell suspension, cells do not reaggregate. If the aggregation factor of one species is then added to the suspension, the aggregates of cells of only this species are formed. Similar experiments were performed by Spiegel (1954) with a mixed suspension of *C. prolifera* and *Cliona celata* (Grant, 1826) cell, which also exhibit a highly species-specific reaggregation. However, Spiegel in his experiments did not remove the aggregation factors from the mixed cell suspension but blocked them with immune sera. The addition of immune serum against the aggregation factor of one species stopped the reaggregation of cells of only this species, without affecting the reaggregation of cells of the other species.

These data and the results of similar experiments performed by other researchers (Moscona, 1968; Leith, 1979) indicated the species-specific nature of action of the aggregation factor, suggesting its involvement in the species-specific reaggregation of sponge cells. These assumptions were fully confirmed in the experiments with colored synthetic granules, which were linked to the aggregation factors of three sponge species (*C. prolifera*, *H. panicea*, and *C. celata*). In the presence of Ca^{2+}, species-specific aggregation of granules was observed in this experimental system, which unambiguously testified to the key role of the aggregation factor in the tissue-specific reaggregation of sponge cells (Popescu and Misevic, 1997; Fernández-Busquets and Burger, 1999; Fernández-Busquets, 2008).

However, in several experiments with mixed cell suspensions, cells of different species are not separated completely, and mixed aggregates of different structure are formed (Curtis, 1962; Humphreys, 1970; Leith, 1979). This indicates that, at least in some sponges, the species specificity of reaggregation is not expressed so strongly. In experiments with several sponge species, Curtis (1962) observed the whole range of possible results of reaggregation in a mixed cell suspension—from complete separation of cells of different species to formation of aggregates in which cells of two species were randomly mixed. Complete separation of cells of two sponge species was observed if the rate of reaggregation of these species differed significantly. When reaggregation rates were similar, the formation of mixed aggregates in a mixed suspension of two species was observed. These data suggest that the species specificity of cell reaggregation is determined not only by the species specificity of action of the aggregation factor but also by the temporal component.

In different clonal lines of the freshwater sponge *E. fluviatilis*, it was shown that reaggregating cells exhibit not only the species specificity but also *individual* specificity. The mixing of cell suspensions obtained from representatives of different clonal lines led to the formation of mixed aggregates at the early stages of reaggregation. However, within several days after mixing, cells in these aggregates were sorted, which led to a complete separation of cells belonging to different lines. That is, aggregates consisting of cells of only one individual were formed (Van de Vyver, 1975; Fernández-Busquets and Burger, 1999). Individual specificity was also retained when cell fractions consisting of only one cell type were mixed (De Sutter and Van de Vyver, 1979; Fernández-Busquets and Burger, 1999).

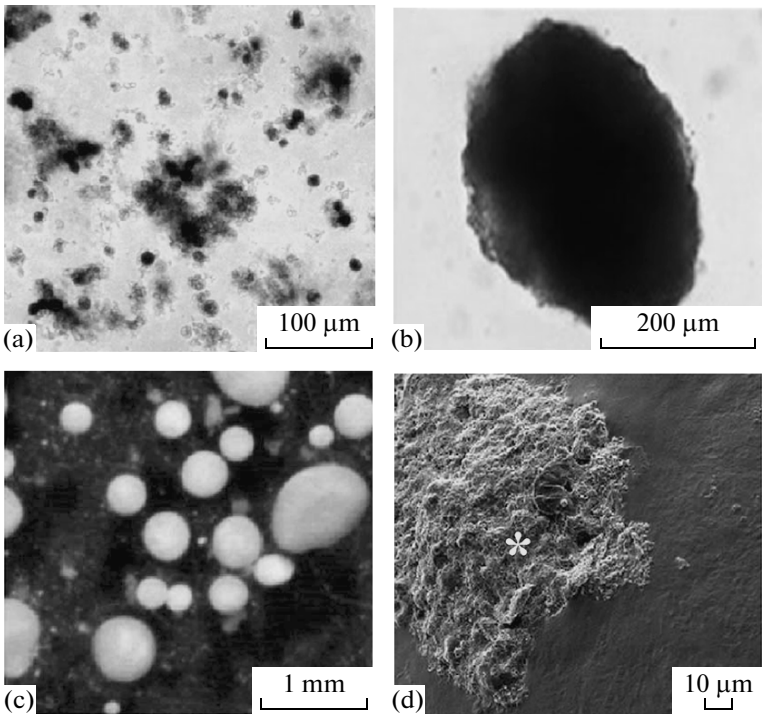

Fig. 4. Main stages of reaggregation of sponge cells under laboratory conditions. (a) Amorphous cell aggregates of *S. massa*, 15 min (by: Sipkema et al., 2003); (b) *L. baicalensis* early primmorph, 6 h (by: Chernogor et al., 2011a); (c) *S. massa* true primmorph, 7 days (by: Sipkema et al., 2003); (d) area of release of detritus and dead cells (marked with an asterisk) on the surface of *S. domuncula* primmorph (by: Sipkema et al., 2003).

Although the mechanism underlying the individual specificity and histocompatibility in sponges remains obscure, some facts indicate that the aggregation factor is involved in this process. (1) In the above-mentioned experiments with *E. fluviatilis*, it was shown that the addition of the aggregation factor of one of the clonal lines to the medium caused enhanced reaggregation of cells of only this line and inhibited this process in cells of other lines (Van de Vyver, 1975). (2) A study of a large number of sponges *C. prolifera* showed that each individual had a different set of genes encoding proteins MAFp3 and MAFp4, which form the core in the aggregation factor molecule. Individual variability was also observed at the level of carbohydrates included in the aggregation factor. This high intraspecific polymorphism is characteristic of the molecular systems involved in allogeneic recognition in higher multicellular animals (Fernández-Busquets and Burger, 1997, 1999; Fernández-Busquets et al., 1998; Fernández-Busquets, 2008). (3) Experiments on allogeneic transplantation in *C. prolifera* showed the accumulation of MAFp3 mRNA and this protein itself in the cells at the boundary of donor and recipient tissues (Fernández-Busquets et al., 1998, 2002).

MAIN STAGES OF CELL REAGGREGATION

The process of sponge cell reaggregation was studied in representatives of two classes—Demospongiae and Calcarea. However, only a small number of studies were performed with the calcareous sponges (Huxley, 1912; Korotkova, 1972). It was shown that the reaggregation rate of cells differed in representatives of Demospongiae and Calcarea at the early stages of reaggregation. For example, in *L. complicata* and *S. lingua* (class Calcarea), the first *cell aggregates* consisting of 15–30 cells were formed only 2–3 h after the beginning of reaggregation, whereas aggregates of the same size were formed as early as 15–40 min after the beginning of reaggregation in sponges belonging to class Demospongiae. In all cases, the first cell aggregates were small and irregular in shape (Fig. 4a). Their size then increased due to the fusion of aggregates with each other as well as due to the joining of single cells from the suspension. Later (2–4 h), cell aggregates of representatives of the class Demospongiae reached a size of hundreds of micrometers (in some cases, 1–1.5 mm), whereas the sponges of class Calcarea reached the same size of aggregates within 12–24 h (Korotkova, 1972; Sipkema et al., 2003; Zhang et al., 2003a; Chernogor et al., 2011), possibly due to a lesser amoeboid motility in suspension.

On the second and third day, aggregates with a spherical shape and a rough surface emerged in cultures (Fig. 4b). This reaggregation stage can be described as *early primmorphs*, which are then gradually transformed into true *primmorphs* (Fig. 4c). The transformation of early primmorphs is accompanied

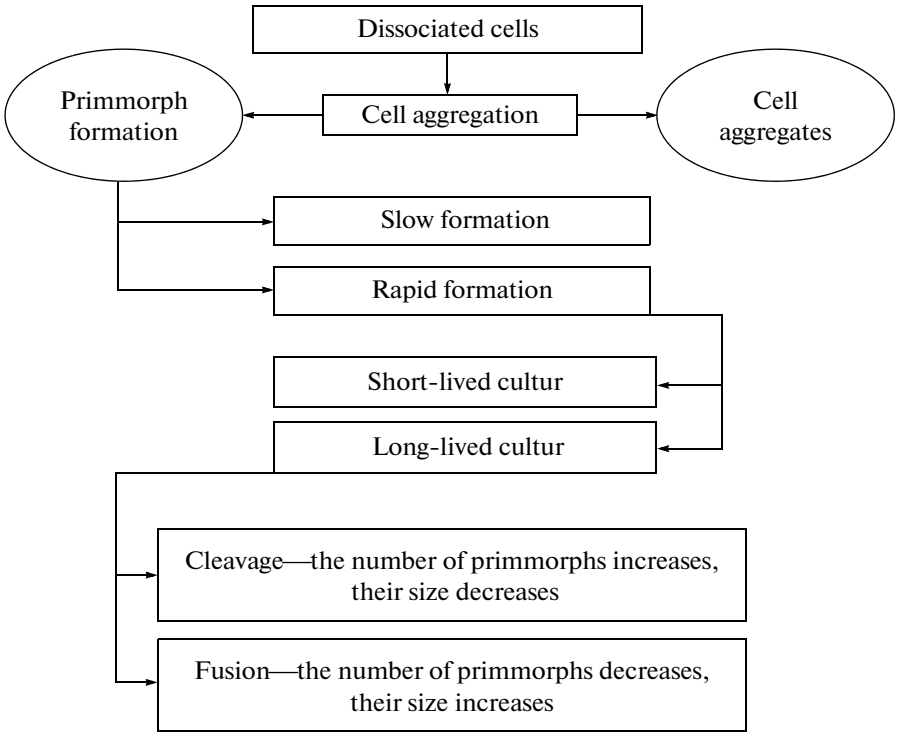

Fig. 5. General scheme of reaggregation of sponge cells in laboratory (by: Valisano et al., 2006a).

by compaction of cell aggregates and formation on their surface of a continuous exopinacodermal layer (in some cases, the first stages of exopinacoderm formation are observed several hours after the beginning of reaggregation) (Müller et al., 1999; Sipkema et al., 2003; Zhang et al., 2003a; Chernogor et al., 2011) (Fig. 7b). During the transformation of primary cell aggregates into primmorphs, they are surrounded with a zone containing detritus, dead cells, and spicules, probably secreted by the aggregates themselves (Müller et al., 1999; Sipkema et al., 2003; Lavrov and Kosevich, 2013) (Fig. 4d). Furthermore, at this time, polysaccharide and collagen can be synthesized de novo in cell aggregates, as was shown for *Hymeniacidon heliophila* (Parker, 1910). In cell aggregates of this species, polysaccharides are synthesized most actively in the first day of reaggregation and drops almost to zero at the time of formation of primmorphs (days 4–5). According to the authors, these results suggest that polysaccharides play an important role in cell adhesion in sponges, particularly at the early stages of cell reaggregation (Vilanova et al., 2010). Changes in the intensity of collagen synthesis during the development of aggregates have not been studied yet.

Studies of a number of species of Mediterranean sponges belonging to the class Demospongiae showed that primmorphs formed on days 3–7 in 14 species, on days 8–20 in five species, and on days 35–40 in one species. In another eight species, primmorphs were not formed: aggregation proceeded only to the stage of cell aggregates (table) (Sipkema et al., 2003; Valisano et al., 2006a). The time of primmorph formation varies in different species, mainly due to the differences in the rate of transformation into true early primmorphs (Sipkema et al., 2003). The lifetime of primmorphs of different sponge species in culture also varies greatly: from 6 days to 10 months (Müller et al., 1999; Sipkema et al., 2003; Valisano et al., 2006a; Chernogor et al., 2011a) (table). The fact that, in some sponge species, reaggregation of cells does not lead to the formation of primmorphs, is explained by anatomical and ecological characteristics of these species. It was assumed that, in sponges of the genus *Cliona*, the formation of primmorphs can be prevented by the sedi-

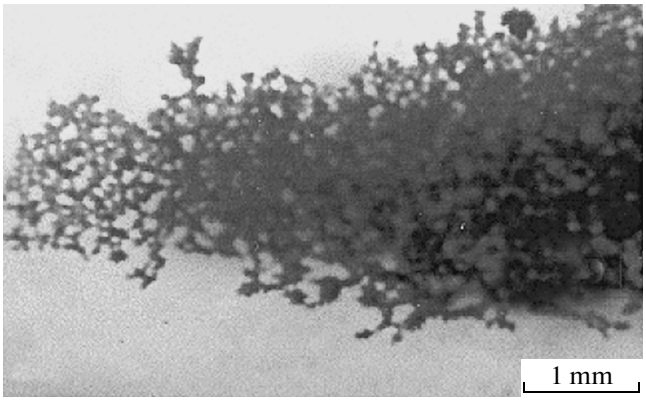

Fig. 6. Attached reticular primmorphs of *D. avara*, 10 days (by: Müller et al., 2000).

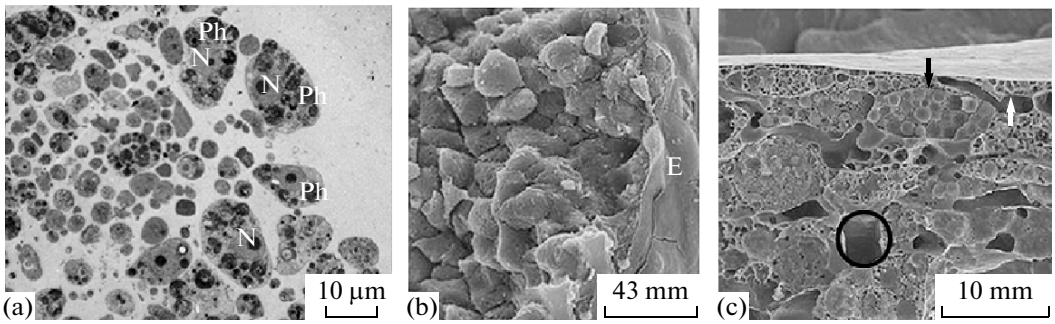

Fig. 7. Histological structure of cell aggregates and primmorphs of sponges. (a) Section of *H. panicea* cell aggregate, 72 h ((N) cell nuclei, (Ph) phagosomes) (TEM) (by: Lavrov and Kosevich, 2013); (b) the outer part of *L. baicalensis* primmorph, 19 days (SEM) (by: Chernogor et al., 2011a); (c) split of *S. massa* primmorph, 7 days ((white arrow) pinacocyte, (black arrow) granulocyte, (black circle) intercellular space (ISS) (according to Sipkema et al., 2003)).

mentary material that accumulates in choanocyte chambers of these sponges as a result of boring activity; in *Aplysina aerophoba*, the formation of primmorphs is prevented by a huge amount of symbiotic bacteria (Valisano et al., 2006a). However, a clear rationale of these assumptions is absent in this study.

Valisano et al. (2006a), who studied reaggregation in a number of species of Mediterranean sponges, indicate that primmorphs derived from different sponge species vary in shape, size, and the total number in culture, even if the same volume of tissue was used for their formation. They also found no correlation between the consistent patterns of primmorph formation and the taxonomic position of the sponge. Such conclusions do not seem entirely correct, because the reaggregation dynamics depends primarily on the concentration of the cell suspension obtained after dissociation of sponge tissues rather than on the amount of tissues. Unfortunately, in their article, the authors do not indicate the concentration of cells in suspensions. However, they assumed that the differences in the number and size of primmorphs can be explained by different "behavior" of primmorphs after their final formation: (1) primmorphs are fused, as a result of which their number decreases and the size increases; (2) primmorphs are cleaved, as a result of which their number increases and the size decreases (Valisano et al., 2006a). All these data and arguments suggest the existence of several pathways of reaggregation of sponge cells.

In general, the reaggregation dynamics of cells can be represented as follows (Valisano et al., 2006a) (Fig. 5):

(1) Dissociated cells;

(2) Cell aggregates;

(3) Primmorphs:

(3.1) Slow formation;

(3.2) Rapid formation:

(3.2.1) Short-lived culture;

(3.2.2) Long-lived culture:

(3.2.2.1) Cleavage (the number of primmorphs increases but their size decreases);

(3.2.2.2) Fusion (the number of primmorphs decreases but their size increases).

A very special type of reaggregation was shown for the sponge *Dysidea avara* (Schmidt, 1862). After the formation of true primmorphs, reaggregation continued, and primmorphs began to fuse on day 10 of cultivation forming a reticulum that adhered to the bottom of the Petri dish (Fig. 6). This reticulum was 10–30 × 5–20 mm in size, the thickness of "stolons" was approximately 50 μm, and the size of pores was approximately 150 μm (Müller et al., 2000). Unfortunately, the further fate of this type of aggregates was not traced.

HISTOLOGICAL STRUCTURE OF CELL AGGREGATES AND PRIMMORPHS

Cell aggregates formed in the first hours after dissociation include all types of cells present in the suspension. At the initial stage, cell comprising the aggregates are loosely packed. The shape of cells is close to spherical; more rarely, they form cytoplasmic processes to interact with neighboring cells. The cytoplasm of some cells contains phagosomes. The cells located on the surface of aggregates do not differ in the morphology from the deeper lying cells (Fig. 7a). These aggregate may also include the fragments of spicules, spongin bands, and symbiotic bacteria and unicellular algae that were present in the tissues of the parent sponge (Efremova, 1969; Korotkova, 1972; Volkova and Zolotareva, 1981; Chernogor et al., 2011). It remains unclear what allows the cells to stick together: the usual fixation methods used in electron microscopy did not reveal any extracellular matrix, and visible contact points between the cells are apparently insufficient.

The transformation of cell aggregates into primmorphs is accompanied by changes in their structure. These changes occur primarily in the surface cell layer:

Time of formation and existence of primmorphs of different sponge species in culture (by: Sipkema et al., 2003, Valisano et al., 2006a, Chernogor et al., 2011 as amended)

Sponge species	Primmorph formation time, days	Lifetime of primmorphs in culture	Source
Acanthella acuta Schmidt, 1862	3	27 days	Valisano et al., 2006a
Agelas oroides (Schmidt, 1864)	15	27 days	Valisano et al., 2006a
Aplysma aerophoba Nardo, 1833	–	–	Valisano et al., 2006a
Axinella damicornis (Esper, 1794)	3	27 days	Valisano et al., 2006a
Axinella polypoides Schmidt, 1862	35–40	>5 months	Sipkema et al., 2003
Axinyssa aurantiaca (Schmidt, 1864)	3	9 days	Valisano et al., 2006a
Batzella inops (Topsent, 1891)	3	27 days	Valisano et al., 2006a
Chondrilla nucula Schmidt, 1862	–	–	Valisano et al., 2006a
Chondrosia reniformis Nardo, 1847	–	–	Valisano et al., 2006a
Clathria prolifera (Ellis & Solander, 1786)	1–2	rs (6–20 days)	Wilson, 1907 Galtsoff, 1925b
Cliona celata Grant, 1826	3	6 days	Valisano et al., 2006a
Cliona nigricans (Schmidt, 1862)	–	–	Valisano et al., 2006a
Corticium candelabrum Schmidt, 1862	–	–	Valisano et al., 2006a
Dysidea avara (Schmidt, 1862)	3	27 days	Valisano et al., 2006a
Ephydatia fluviatilis (Linnaeus, 1759)	1	rs (6–7 days)	Efremova, 1969
Geodia cydonium (Jameson, 1811)	7–10	>2 months	Sipkema et al., 2003
Halichondria panicea (Pallas, 1766)	3–20	1–5 months	Korotkova, 1972
		rs (25–30 days)	Sipkema et al., 2003 Lavrov and Kosevich, 2013
Haliclona aquaeductus (Shmidt, 1862)	5–8	1–2 months	Lavrov and Kosevich, 2013
Haliclona fulva (Topsent, 1893)	3	27 days	Valisano et al., 2006a
Haliclona oculata (Pallas, 1766)	18	18 days	Sipkema et al., 2003
Halisarca dujardini Johnston 1842	2–3	rs (20–25 days)	Volkova and Zolotareva, 1981; Kuternitskaya et al., 2008
Hemimycale columella (Bowerbank, 1847)	3	15 days	Valisano et al., 2006a
Hymeniacidon agminata Ridley, 1884	5–7	N/D	Zhang et al., 2003a
Hymeniacidon perlevis (Montagu, 1818)	5	N/D	Zhang et al., 2003b
Hymeniacidon heliophila (Parker, 1910)	4–5	N/D	Vilanova et al., 2010
Ircinia oros (Schmidt, 1864)	–	–	Valisano et al., 2006a
Leucosolenia complicata (Montagu, 1818)	5–6	rs (3 weeks)	Korotkova, 1972
Lubomirskia baicalensis (Pallas, 1766)	2	10 months	Chernogor et al., 2011
Petrosia ficiformis (Poiret, 1789)	3	27 days	Valisano et al., 2006a
Phorbas fictitious (Bowerbank, 1866)	3	15 days	Valisano et al., 2006a
Pleraplysilla spinifera (Schulze, 1879)	3	27 days	Valisano et al., 2006a
Pseudosuberites aff. *andrewsi*	4–8	9 days	Sipkema et al., 2003
Spirastrella cunctatrix Schmidt, 1868	3	27 days	Valisano et al., 2006a
Spongia officinalis Linnaeus, 1759	–	–	Valisano et al., 2006a
Stylissa massa (Carter, 1887)	7–10	>2 months	Sipkema et al., 2003
Suberites domuncula (Olivi, 1792)	3–7	4–5 months	Custudio et al., 1998 Valisano et al., 2006a
Sycon lingua (Haeckel, 1870)	2–3	rs (4 weeks)	Korotkova, 1972
Sycon raphanus Schmidt, 1862	5	rs (17–30 days)	Huxley, 1912

(Dash) primmorphs were not formed in the experiments, (N/D) the lifetime of primmorphs in cultures was not determined, (rs) the regeneration of functional sponges in primmorph cultures was observed (the time of complete regeneration of sponge is shown in parentheses).

cells become flattened or T-shaped and gradually form a continuous exopinacodermal layer on the primmorph surface (Figs. 7b, 7c). At this time, the distribution of collagen fibers inside the aggregate also changes. In the primary (24 h) cell aggregates, collagen fibers are scarce and distributed randomly. However, by the time of primmorph formation, the collagen fibrils fill the intercellular space and form a continuous layer on the surface of primmorphs, which probably ensures mechanical support of the exopinacocyte layer (Vilanova et al., 2010). On the surface of *E. fluviatilis* primmorphs, unlike other sponge species, a double layer of pinacocytes is formed, which is partly separated from the inner cell mass and forms hypodermal cavities (Efremova, 1969).

The cells of the inner mass of primmorphs gradually become fairly densely packed. They have an amoeboid shape, and most of them can be defined as amoebocytes and archaeocytes. More rarely, the inner mass may also contain lophocytes (Vilanova et al., 2010) or various cells with inclusions, whose ultrastructure is reminisce of the ultrastructure of cells in intact sponge tissues (Korotkova, 1972; Custudio et al., 1998; Müller et al., 1999; Le Pennec et al., 2003; Chernogor et al., 2011) (Fig. 7c). Despite the dense packing of cells in primmorphs, electron microscopic analysis shows that the cytoplasmic membranes of adjacent cells are arranged parallel to each other and are separated by a small distance.

CHARACTERISTICS OF PHYSIOLOGY OF PRIMMORPHS

Studies have shown that the physiological state of cells comprising the cell aggregates and primmorphs differs from that in intact sponge tissues.

The study of the level of telomerase activity in *Suberites domuncula* (Olivi, 1792) using the Telomerase Detection Kit (TRAP) showed that, in primmorphs, it is fairly high as compared to the cells in suspension but much lower than in the cells of intact sponge tissues. In intact tissues, telomerase activity was 8.9 units of TPG (total product generated) per 5×10^3 cells. In the cells that were present in suspension for 14 h, telomerase activity dropped to 0.9 TPG units per 5×10^3 cells. In primmorphs (day 10 after formation from individual cells), telomerase activity increased to 4.7 TPG units per 5×10^3 cells (Custudio et al., 1998; Müller et al., 1999).

The detection of telomerase activity in the cells comprising primmorphs indicates that they retain the ability to undergo mitosis. Studies of mitotic activity in *S. domuncula* at different stages of reaggregation using the BrdU label (5-bromo-2'-deoxyuridine) showed that the cells that were present in suspension for 24 h did not incorporate the label in their nuclei. Cell aggregates obtained after 24 h of culturing contained 6.5% of BrdU-positive cells. In primmorphs, the number of BrdU-positive cells depended on the primmorph age. For example, the number of BrdU-positive cells in 3-day and 1-month primmorphs was 33.8 and 22.3%, respectively. These data indicate that the cells in *S. domuncula* primmorphs synthesized DNA and probably underwent cell divisions (Custudio et al., 1998; Müller et al., 1999).

The ability of primmorph cells to synthesize DNA and, possibly, undergo mitosis indicates that these cell aggregates have prerequisites for growth and progressive development. However, observations showed that, during long-term cultivation of primmorphs of *Petrosia ficiformis* (Poiret, 1789), their biomass did not increase. The dry weight of the primmorphs after 20 days of culturing was approximately ten times smaller than the dry weight of the sponge body part of the same volume that was used to obtain these primmorphs. In this experiment, primmorphs were cultured in filtered seawater without adding any nutrients (Valisano et al., 2006b).

Primmorphs, similarly to normal sponges, are able to synthesize biologically active substances. After 3–6 days of cultivation, extracts of *D. avara* primmorphs contained significant quantities of avarol (0.4–0.9 μg per 100 μg of tissue), which accounts for approximately 30% of the content of avarol in intact sponge tissues. The content of avarol in cells significantly increased after forming reticular primmorphs and reached 1.4 μg per 100 μg of tissues (80% of the avarol content in sponge tissues) (Müller et al., 2000).

Under certain culturing conditions and during progressive development leading to the regeneration of the original organization of sponges, active synthesis of new spicules in primmorphs was observed, whose shape and dimensions were typical for the given sponge species (Le Pennec et al., 2003; Valisano et al., 2007a; Chernogor et al., 2011b).

MORPHOGENETIC POTENTIAL OF PRIMMORPHS

Despite the 100-year history of studies of cell reaggregation in sponges, the views of researchers on the morphogenetic potential of primmorphs vary greatly.

The early studies of cell reaggregation in sponges (Wilson, 1907; Huxley, 1912; Galtsoff, 1925b) described complete regeneration of functional individuals of *C. prolifera* and *Sycon raphanus* (Schmidt, 1862) from cell suspensions obtained by the mechanical method. Spherical cell aggregates formed as a result of reaggregation were able to fuse and attach to the substrate. After attachment, the formation of the canals the water-current system (they appeared as isolated lacunas that gradually fused with each other), choanocyte chambers, and skeletal elements in the aggregates was observed. The final stage of the reorganization of aggregates was the appearance of an oscular elevation. In thus obtained sponges, water currents

and beating of choanocyte flagella were observed. The entire process of sponge regeneration in the laboratory took 6–20 days.

Conversely, a number of modern authors (Krasko et al., 2002; Wiens et al., 2003) indicate a limited morphogenetic potency of primmorphs and the impossibility to restore a functional sponge after dissociation and reaggregation of its cells. However, it was shown that the cultivation of *S. domuncula* primmorphs on polylysine or galectin substrates (Wiens et al., 2003; Müller et al., 2004b), exposure to a strong current (20 cm/s) (Perovic et al., 2003), or the addition of iron and silicon ions to the culture medium (Le Pennec et al., 2003) led to the formation of canals in the water-current system and dermal membrane in the primmorphs. However, further progressive development of aggregates in these experiments was not observed.

Researchers also differ in opinion regarding the processes occurring during the morphogenetic transformations of primmorphs. These differences are associated with the possibility and degree of dedifferentiation of cells after their dissociation and with the possibility of transdifferentiation and involvement of different cell types in the formative processes. For example, Huxley (1912), Brien (1937), and Brønsted (1937) considered the developmental processes that occur after the dissociation of tissues as "self-assembly" and rearrangement of cells in accordance with their original differentiation. According to Huxley (1912), after the dissociation of tissues, cells temporarily dedifferentiated; however, their redifferentiation could proceed only in the original direction. According to the standpoint of the above-mentioned authors, a new sponge can develop only from those primmorphs that contain all cell types present in an intact sponge. Another point of view was held by Wilson (1907) and Galtsoff (1925b), who allowed for the possibility of transdifferentiation of certain types of cells and these cells giving special importance in morphogenesis. Wilson (1907) believed that, after tissue dissociation, all cells undergo dedifferentiation and return to the embryonic totipotent state. Galtsoff (1925b), conversely, denied the possibility of dedifferentiation of cells. He regarded the changes occurring in cells after tissue dissociation only as slight changes in morphology but not as dedifferentiation of cells.

Detailed studies of reaggregation of sponge cells, performed in the Laboratory of B.P. Tokin at Leningrad State University in the 1970–1980s, allowed some of these questions to be answered. It was shown that the formation and progressive development of primmorphs was accompanied by the death of some cells as well as by dedifferentiation and transdifferentiation of certain cell types. During the primmorph development, viable cells either retain their differentiation or, in some cases, can undergo transdifferentiation into other cell types. In the representatives of classes Demospongiae and Calcarea, transdifferentiation is observed primarily in nucleolar amoebocytes and choanocytes, respectively. These cell types first undergo dedifferentiation, after which they become able to transdifferentiate to exopinacocytes and endopinacocytes, choanocytes, sclerocytes, and collencytes. This is a way to compensate for the deficit of these types of cells. At the same time, endopinacocytes and amoebocytes with specific inclusions retain their original differentiation (Efremova, 1968, 1969, 1972; Efremova and Nikitin, 1974; Nikitin, 1974; Volkova and Zolotareva, 1981; Korotkova, 1997).

In determining the fate of cells undergoing transdifferentiation, the space occupied by them in the primmorph plays a major role. Groups of choanoblasts always occur in the central part of the primmorph, whereas pinacocytes emerge predominantly on or near the surface. Apparently, a certain physiological gradient occurs in the primmorph, associated with the occurrence of differences in the microenvironment in the center and at the periphery of aggregate, which determine the direction of cell transdifferentiation (Nikitin, 1974; Volkova and Zolotareva, 1981). This assumption is confirmed by the inability of aggregates that are smaller than 150 μm to undergo progressive development. The surface of such aggregates is epithelized due to pinacocytes and never due to choanoblasts; this phenomenon can be explained by insufficient differences in the microenvironment in the center and at the periphery of the aggregate due to its small size (Efremova and Nikitin, 1974; Nikitin, 1974; Korotkova, 1979, 1997).

Studies in the same laboratory also confirmed that reaggregation of cells of different sponge species proceeds with different intensity and is not always completed by the formation of viable cell aggregates. There is a correlation between the reaggregation pattern of cells in a given sponge species and its lifecycle structure (Korotkova, 1979). It is known that the anatomical structure and tissue organization of sponges may undergo serious seasonal changes during the lifecycle (Korotkova, 1979; Ereskovsky, 2000). If the lifecycle of the sponge after sexual reproduction includes the stage of formation of internal buds or reductive bodies, the reaggregation of cells of this species usually leads to the restoration of the original organization of the animal. Examples of such species are the freshwater sponges of the genera *Ephydatia* and *Spongillla*, as well as representatives of the marine genera *Halisarca* and *Halichondria* (Korotkova, 1979). It was shown that cell aggregates of *E. fluviatilis* (Efremova, 1969, 1972), *S. lacustris* (Efremova, 1972), *H. dujardini* (Volkova and Zolotareva, 1981), *H. panicea*, *L. complicata*, and *S. lingua* (Korotkova, 1972) are capable of progressive development, which is completed by the formation of a new sponge. During the development, the aggregates of these species pass through the stages similar to those described for the aggregates of *C. prolifera* and *S. raphanus* (Wilson, 1907; Huxley, 1912; Galtsoff, 1925b). The

aggregates of *E. fluviatilis* developed to normal sponges within 6–7 days. The aggregates of *H. dujardini*, *H. panicea*, *L. complicata*, and *S. lingua* developed more slowly: fully formed sponges emerge only 20–30 days after the beginning of experiments. This may be due to the fact that these experiments were conducted at lower temperatures, because they were performed with the cold-water representatives of the above-mentioned species from the White Sea.

In general, the process of formation of a new sponge during progressive development of aggregates includes the following stages: (1) cell reaggregation and formation of stable aggregates (early primmorphs) of a certain size, (2) epithelization of the surface of early primmorphs (transformation into real primmorphs), (3) attachment to the substrate, and (4) acquisition of a definitive organization (formation of the water-current system, skeleton, etc.) (Efremova, 1969; Korotkova, 1972, 1997; Volkova and Zolotareva, 1981; Korotkova, 1979).

In the majority of sponge species, the attachment of primmorphs to the substrate is the key factor in development (Galtsoff, 1925b; Volkova and Zolotareva, 1981). At this moment, the type of symmetry changes from polyaxial for apicobasal. The attachment to the substrate is accompanied by significant changes in the distribution of different types of cells in the primmorph. If a developing primmorph is separated from the substrate, regressive changes in his organization are observed (Efremova, 1969; Korotkova, 1972; Efremova and Nikitin, 1974; Galtsoff, 1925b). However, unlike other sponge species, the attachment of *H. dujardini* to the substrate is not the crucial factor in its development. Primmorphs of this species can grow in suspension. However, the absence of contact with the substrate affects the final stage of development—the formation of osculum. In unattached primmorphs, not one but several ocscular openings are formed (Volkova and Zolotareva, 1981).

The above facts clearly testify to the possibility of restoring the original organization of the sponge as a result of reaggregation of its cells. It can be postulated with a high probability that, during reaggregation of cells and subsequent sponge formation, the processes associated with dedifferentiation and transdifferentiation of cells as well as with different behavior of cells depending on their position in the primmorph are of principal importance. This statement makes it possible to completely abandon the understanding of the processes occurring after the dissociation of sponge tissues as a "self-assembly" of initially differentiated cells.

FACTORS INFLUENCING THE PROCESS OF REAGGREGATION

The process of reaggregation of cells, as well as the size of cell aggregates, their number, structure, and physiology depend on many different factors.

Physiological state of the sponge. The physiological state of the sponge whose cellular material is used in experiments significantly affects the process of reaggregation. A long-term (more than 14 days) keeping of a sponge in an aquarium before experiments has an adverse effect on cell reaggregation. As a result, only small cell aggregates incapable of further progressive development and dying within a few days are formed in the suspensions of cells derived from such animals (Galtsoff, 1925a; Valisano et al., 2006b). This phenomenon can be explained by the general deterioration of the state of sponges during long-term keeping in simple aquarium systems.

An important factor is also the stage of the lifecycle of the sponge. It was shown for *E. fluviatilis* that reaggregation proceeds most effectively in the suspensions of cells derived from those animals that have just started sexual reproduction or gemmulogenesis. If individuals that already actively form gemmules are used in experiments, the initial stages of cell reaggregation proceed very rapidly; however, the morphogenetic processes that are associated with the attachment to the substrate and formation of the water-current system are decelerated later. When cellular material of young sponges is used, only very small aggregates consisting primarily of choanocytes incapable of further development are formed in cell cultures (Efremova, 1969, 1972; Korotkova, 1997).

Cryopreservation of cell suspension. In most cases, experiments on sponge cell reaggregation should be performed with a freshly prepared cell suspension. However, cryopreserved cells can also be used. Using cell suspensions derived from several species of class Demospongiae, it was shown that cryopreservation only slightly reduces the viability of cells. For example, freshly prepared cell suspensions of the studied species contained 80–100% of live archaeocytes. After cryopreservation in 15% DMSO, the amount of live archaeocytes was reduced to 70–95% (Pomponi, 2006).

Cellular composition of suspension. A suspension of cells obtained as a result of dissociation of sponge tissues can be divided into sized fractions by centrifugation in stepwise or continuous density gradients. Sized fractions represent either pure fractions of cells of a certain type or fractions enriched in a certain cell type.

For example, the fractionation of cell suspension of the marine sponge *Hymeniacidon perlevis* (Montagu, 1818) allows for obtaining three fractions of cells: C_1—fraction enriched in small cells (cells smaller than 2 μm in diameter account for 80% of this fraction); C_2—fraction containing a mixture of cells of all size classes; and C_3—fraction enriched in large cells (76% of this fraction is constituted by cells 10–15 mm in diameter, which can be characterized as archaeocytes and archaeocytes-like cells) (Zhang et al., 2003b). Attempts to obtain primmorphs from cells of individual fractions showed that only the fraction of

the large cell (C_3) was able to form primmorphs. When this fraction was used in experiments, primmorphs were formed already on the second day, whereas primmorphs formed only on day 4–5 in the conventional cell suspension obtained from the same sponge. In addition, primmorphs obtained from the fraction of large cells (C_3) had a more regular and smooth shape and showed a higher level of DNA synthesis that the conventional primmorphs throughout the experiment (Zhang et al., 2003b) (Fig. 8).

In the suspension of cells of the freshwater sponge *E. fluviatilis*, the following fractions of cells can be distinguished (De Sutter and Van de Vyver, 1977, 1979; Buscema et al., 1980): A_1—pure fraction of archaeocytes; B_{2+3}—fraction comprising all types of sponge cells except for archaeocytes; C_2—fraction enriched in choanocytes; and C_3—fraction comprising all types of sponge cells, except for archaeocytes and choanocytes.

The reaggregation of cells in fraction A_1 leads to the formation of primmorphs 24 h after the beginning of experiment and further regeneration of the original organization of the sponge in 2–3 days (De Sutter and Van de Vyver, 1977; Buscema et al., 1980). The authors of these studies did not compare the reaggregation rates in this fraction and in the conventional cell suspension obtained from the same individual. However, data of Efremova on the reaggregation rate of a normal cell suspension suggest that, similarly to *H. perleve*, the archaeocyte fraction of *E. fluviatilis* cells exhibits an accelerated reaggregation rate (Efremova, 1969).

In the B_{2+3} fraction, reaggregation reaches only the stage of primmorphs. Further regeneration of the sponge is not observed. Cells in the C_2 fraction aggregated more rapidly compared to the cells of other fractions; however, the process of reaggregation goes only to the stage of cell aggregates, which die several days after their formation. The C_3 fraction has no ability to reaggregate (De Sutter and Van de Vyver, 1977, 1979).

To study the effect of the cellular composition of suspension on the process of cell reaggregation, Korotkova (1979, 1997) performed assembly of cell aggregates using a micromanipulator. As a result of these experiments, artificial cell aggregates that contained only one type of sponge cells were obtained. It was shown that the aggregates consisting of only pinacocytes or choanocytes cannot be transformed into a functional sponge. Aggregates comprised of archaeocytes exhibit a greater morphogenetic potential and are able to restore the original organization of the animal within 6–7 days.

These data indicate that different types of sponge cells possess different morphogenetic potentials and ability to undergo dedifferentiation and transdifferentiation and induction of regenerative processes.

Concentration of cells in suspension. Based on general considerations, we can assume that the concentration of cells in suspension should influence the process of reaggregation. Indeed, the study of *S. domuncula* showed that the use of a larger initial concentration of cells leads to the formation of larger primmorphs. For example, a threefold increase in the concentration of cells in suspension (from 2×10^7 cells/mL to 6×10^7 cells/mL) led to an almost threefold increase in the size of primmorphs (Sipkema et al., 2003). In *Hymeniacidon agminata* (Ridley, 1884), an increase in cell concentrations leads to an increase in not only the size of primmorphs but also the rate of their formation (Zhang et al., 2003a). The effect of cell concentration in the initial suspension on the formation rate and size primmorphs should be primarily manifested in those sponge species whose separated cells do not possess amoeboid motility.

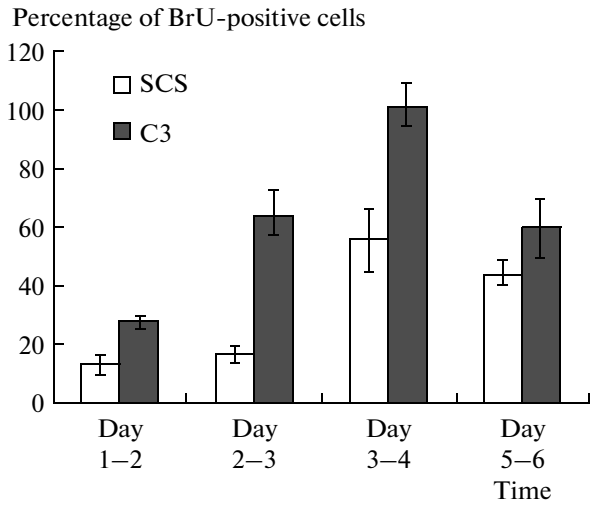

Fig. 8. Relative number of BrdU-positive cells at different stages of reaggregation of *H. agminata*. Designations: (SCS) standard cell suspension; (C3) C_3 cell fraction enriched in archaeocytes and archaeocytes-like cells. Bars show the standard deviation of six replicates (by: Zhang et al., 2003b).

Temperature. Effect of temperature on the reaggregation of sponge cells is ambiguous. For the suspensions of cells of *H. agminata* (species from the South China Sea; these sponges were kept in an aquarium at 24–26°C before the experiments), a high temperature is optimal. At 30°C, primmorphs of this sponge species emerged as early as 24 h after the beginning of experiment; this process took 2–3 days at 25°C and primmorphs were not formed at 15°C (Zhang et al., 2003a).

Opposite results were obtained in the studies performed with the primmorphs of *P. ficiformis* (species from the Mediterranean Sea; these sponges were kept in an aquarium at 12°C before the experiment) and *L. baicalensis* (species from Lake Baikal; these sponges were kept in an aquarium at 3–4°C before experiments). In these cases, low temperature was optimal for reaggregation. For example, in *P. ficiformis* cultures at 12 and 24°C, the size of primmorphs was

0.1 and 0.04 mm^2, respectively. In addition, in the first week of culturing at 24°C, a high mortality of primmorphs was observed (Valisano et al., 2007b). For *L. baicalensis*, the optimum temperature was 3–6°C. At this temperature, active formation of primmorphs was observed, and they retained viability for a long time. At 12°C, primmorphs were formed more slowly; in addition, many primmorphs died several days after the beginning of experiments. At 18°C, reaggregation of *L. baicalensis* cells was observed only in the first few hours, after which the cells died (Chernogor et al., 2011).

Apparently, the temperature, that optimal for the process of reaggregation of cells, is directly related to the environmental conditions in which the relevant species inhabit sponges.

Culture medium. Sponge cells are usually cultured in fresh or sea water sterilized by filtration. For successful long-term culturing of primmorphs, the culture medium is often supplemented with antibiotics and fungicides to prevent excess growth of bacteria and fungal infections, which have an adverse effect on reaggregation of sponge cells. However, different antibiotics and fungicides have different effects on primmorph cultures. For example, the addition of penicillin, gentamicin, rifampicin, streptomycin, and penicillin–streptomycin mixture to the culture medium did not significantly affect cell reaggregation (Sipkema et al., 2003; Pomponi, 2006). However, the addition of a mixture of four antibiotics (kanamycin, tylosin, tetracycline, and gentamicin) inhibited the formation primmorphs in seven Mediterranean sponge species (*S. domuncula*, *G. cydonium*, *S. massa*, *Pseudosuberites* aff. *andrewsi*, *H. panicea*, *H. oculata*, and *A. polypoides*). In the presence of these antibiotics, only amorphous cell aggregates are formed. Possibly, the adverse effect in this case was associated with too high concentration of antibiotic in the mixture: the concentrations of tylosin and tetracycline were exceeded ten times in comparison with the manufacturer's recommendations (Sipkema et al., 2003).

These data are of additional interest with allowance for the fact that all sponge species form tight and specific symbiotic associations with bacteria. Many sponges are inhabited with a complex microbial community, which can account for up to 40% of the body weight of the animal. Symbiotic bacteria are involved in the utilization of metabolic products of sponges, provide them the nutrients, and produce secondary metabolites for chemical defense of the host against predators and fouling organisms (Kuternitskaya et al., 2008; Haygood et al., 1999; Lee et al., 2001; Thakur et al., 2003). It was also shown that many metabolites, including the potential biologically active substances, are the products of joint activity of sponge cells and symbiotic bacteria (Müller et al., 2004a).

Possibly, the elimination of symbiotic bacteria from cell suspension after the addition of antibiotics to the culture medium may prevent normal reaggregation of cells and further regeneration of the sponge structure. Similar findings were obtained in the studies of reaggregation of *H. dujardini* cell. In cell cultures of this species, reaggregation stops completely if antibiotic ampicillin is added to the culture medium immediately after the preparation of suspension. In such cultures, neither cell aggregates nor primmorphs are formed. If the antibiotic is added 20–30 min after the beginning of cultivation of the cell suspension, the formation of cell aggregates and primmorphs proceeds normally and does not differ from the control (reaggregation of cells in the antibiotic-free medium). According to the authors of that study, this result of the experiments suggests that the initial period of reaggregation of cells is essential for determination of further interaction of sponge cells and their symbiotic bacteria (Kuternitskaya et al., 2008).

The above data indicate that, for a successful long-term culturing of primmorphs, it is necessary to select the optimum set and doses of antibiotics for each sponge species. Attention should be paid not only to the efficiency of the given antibiotic in inhibiting microbial contamination but also on the nature of its effect on sponge cells. In addition, normal functioning of sponge cells is apparently almost impossible without symbiotic bacteria. This fact should also be taken into account in selecting the conditions for long-term culturing of primmorphs and in the experiments related to the regeneration of a normal sponge from cell suspension.

Amphotericin has an adverse effect on cell reaggregation, which was due to the mechanism of action of this fungicide: amphotericin destroys fungal cells by linking to ergosterol, the main sterol in cell membranes. Biochemical studies of several sponge species (*D. avara*, *Ircinia pipette* (Schmidt, 1868), *Spongionella gracilis* (Vosmaer, 1883), and *Tethya aurantium* (Pallas, 1766)) showed that ergosterol is present in the membranes of their cells. Moreover, ergosterol is the major sterol in cell membranes of *D. avara*, *I. pipette*, and *S. gracilis* (Sipkema et al., 2003, 2004).

The selection of a suitable composition of the culture medium can increase the lifespan of cell aggregates and primmorphs in culture and intensify the metabolism of their cells. As a result, this can provide an increase in the biomass of the culture, more rapid development of primmorphs and functional sponges, and increased production of bioactive substances. To date, the majority of studies aimed at creating culture medium for sponge cells were performed with cultures of dissociated cells. Nevertheless, some of these studies were performed with primmorphs.

The effect of the addition of silicon and iron ions to the culture medium was studied in the primmorph cultures of some sponge species. The reason for these experiments was the fact that iron and silicon ions intensify DNA and protein synthesis in sponge cells

and stimulate spiculogenesis (Krasko et al., 2002). However, the results obtained with different sponge species were discrepant. For example, the addition of 60 μM silicon ions to the culture of *S. domuncula* primmorph caused a threefold increase in their size (from 2 to 6 mm). Subsequent addition of 30 μM iron ions caused an additional increase in the size of primmorphs (to 10 mm) and the development of canals of the water-current system (Le Pennec et al., 2003).

At the same time, the addition of 60 μM silicon ions and 30 μM iron ions to the *P. ficiformis* primmorph culture had only a weak effect, and an increase in the concentration of iron ions to 60 μM led to the destruction of aggregates within several weeks. The most pronounced positive effect was caused by the addition to the culture medium of silicon ions alone at concentrations of 120 and 180 μM (Valisano et al., 2007a).

The addition to the culture medium of silicon ions alone or silicon and iron ions promoted spiculogenesis in the primmorphs of the above two species, as well as in the primmorphs of the freshwater sponge *L. baicalensis*. In the case of *P. ficiformis*, not only the number of spicules but also their size increased (Krasko et al., 2002; Le Pennec et al., 2003; Valisano et al., 2007a; Chernogor et al., 2011b).

The mechanism by which iron and silicon ions increase the size of aggregates remains unclear. The development of canals may be determined by the deficit of oxygen when the size of primmorphs increases. The water-current system in this case ensures the delivery of oxygen to the aggregate tissues (Le Pennec et al., 2003). However, data on the baseline concentrations of iron and silicon ions in the original culture medium are absent, and only the effect of added concentrations of these elements was studied.

Studies of the cultures of dissociated cells of marine sponges showed that the addition of pyruvate, vitamin C, sodium chloride, and iron ions to the medium caused a significant increase in cell viability (Zhang et al., 2004; Zhao et al., 2005; Pomponi, 2006; de Caralt et al., 2007). Positive effects on cell viability and metabolism intensity (levels of DNA and protein synthesis and esterase activity) were observed after the addition to the culture medium of certain growth factors and hormones (phytohemagglutinin, insulin, epidermal growth factor, and prostaglandin E2) and cultivation of cells in diluted commercial media that are usually used for the cultivation of mammalian cells (Osinga et al., 1999; Pomponi, 2006; de Caralt et al., 2007).

It is quite obvious that the composition of the culture medium has a significant effect on the process of reaggregation of sponge cells. However, despite the fact that many studies are dedicated to this problem, many results are ambiguous and can vary from one experiment to another or from one object to another.

CONCLUSIONS

In changing hydrodynamic environmental conditions, for effective water filtration through the entire surface and volume of the body, sponges have to constantly rearrange their water-current system. In this regard, tissues, cell lines, and the organization of the water-current system of these animals are extremely ductile (Ereskovsky, 2005). At the same time, despite the constant movement of cells and remodeling of anatomical structures, the sponge is a single integrated organism. In such organization, these animals found a balance between the very high ductility of all structures of the body and the integration of these structures into a single system. This requires the mechanisms of cell–cell integration and communication, which differ significantly from those found in other Metazoa. Reaggregation of sponge cells provides a convenient model system that makes it possible to study various aspects of tissue functioning in sponges under controlled conditions.

Reaggregation of sponge cells includes several stages: (1) cell adhesion and formation of stable multicellular aggregates, (2) epithelization of multicellular aggregates (formation of primmorphs), and, in some cases, (3) development of primmorphs to a completely functional sponge. Different stages of this process make it possible to study the mechanisms of cell adhesion and immune recognition, behavior and interaction of different cell types, as well as to trace the restoration of the initial connections between cells and the formation of the main structural elements of the body.

Sponge cells reaggregate due to the presence of a macromolecular complex—the aggregation factor. Judging by the data accumulated to date, the same complex is involved in providing species and, probably, individual cell and tissue specificity of sponge tissues of the jaws. However, many details of immune recognition in sponges, as well as the molecular mechanisms underlying this process remain obscure (Fernández-Busquets, 2002). The combination of the mechanisms of cell adhesion and immune recognition in sponges makes it possible to use early stages of the reaggregation process for studying the mechanisms of immune recognition at the level of individual cells and cell lines. Due to the relatively simple organization of sponges and the absence of organs in them, it can be assumed that the immune system of these animals is also arranged relatively simply. The study of the immune system in the most primitive Metazoa may help to better understand the structure and details of immune system functioning in higher animals, including humans.

The intensity of cell reaggregation differs not only in different sponge species but also within the same species depending on the physiological state of the animal, which is associated with the lifecycle stages. Many sponges undergo severe tissue reorganization during the lifecycle, as well as degenerative and regen-

erative phenomena associated with sexual and asexual reproduction (Ereskovsky, 2000). Apparently, the morphogenetic potential of dissociated cells and, as a consequence, the reaggregation of cells strongly depends on the initial physiological state of the individual. Consequently, the study of the reaggregation process in a wide range of sponge species at different stages of their lifecycles will make it possible to understand the morphogenetic potential and relationship between cell lines, as well as the changes in the physiological state of cells depending on the lifecycle stage and the changes of the morphogenetic potential of cells associated with this.

Of particular interest is the primmorph stage. After the final formation of these aggregates, the recovery of the initial organization is observed in several sponge species. This process is not the result of a simple "self-assembly" and sorting of differentiated cells. The described regenerative morphogenetic processes are accompanied by active dedifferentiation and transdifferentiation of certain cell types. The subsequent fate of cells is apparently determined by their position in the aggregate. During the development of primmorphs, a new coordinate system is formed, which regulates further processes of restoration of sponge organization. Thus, the formation of primmorphs and the restoration of normal sponges from them makes it possible to study the processes of formation and regulation of the spatial organization of multicellular organisms in the course of their ontogeny. An example of such studies is the investigation of the role of the Wnt signaling pathway in the formation of ectopic osculums in the sponge *Oscarella lobularis* (Lapébie et al., 2009; Winsdor and Leys, 2010; Ereskovsky et al., 2013).

The issues relating to the processes of determining and governing the possibility of restoration of the intact organization of sponges from primmorphs are of special interest. A detailed study of cell behavior during the transformation of primmorphs into a normal sponge will make it possible to understand the mechanisms that ensure the integration of sponge tissues and the high plasticity of cellular and anatomical structures in their body. The role of the extracellular matrix in the regulation of development and functioning of sponges and other metazoans remains insufficiently studied (Aouacheria et al., 2006). It is necessary not only to determine the main components of the extracellular matrix but also to study the mechanisms that regulate its release at different stages of ontogeny of the organism. This will make it possible to analyze the effect of the extracellular matrix on the cell behavior. Primmorphs, as a model system, to some extent facilitate the solution of this problem, because they allow performing studies under controlled conditions, which is difficult in working with intact sponges.

Potentially possible long-term stable primmorph cultures will also make it possible to study the biochemistry and physiology of different cells and the processes of spiculogenesis. In addition, long-term primmorph culture can become the basis for obtaining biologically active substances from sponges for the pharmaceutical and cosmetic industry (Fernández-Busquets, 2002; Pomponi, 2006). However, this requires a serious revision of the methods associated primarily with the selection of the composition of the culture medium and culturing conditions for primmorphs. This is necessary for increasing the lifespan of primmorphs and stimulating mitotic divisions in them, which will ensure a biomass increase in cultures.

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Translated by M. Batrukova