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Transdifferentiation and mesenchymal-to-epithelial transition during regeneration in Demospongiae (Porifera)

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3 1 **Title**¹ Transdifferentiation and mesenchymal-to-epithelial transition during regeneration
4 in Demospongiae (Porifera)
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36 20 **Text figures** – 16

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39 22

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Abstract

Origin and early evolution of regeneration mechanisms remain among the most pressing questions in animal regeneration biology. Porifera have exceptional regenerative capacities and, as early Metazoan lineage, are a promising model for studying evolutionary aspects of regeneration. Here, we focus on reparative regeneration of the body wall in the Mediterranean demosponge *Aplysina cavernicola*. The epithelialization of the wound surface is completed within two days, and the wound is completely healed within two weeks. The regeneration is accompanied with the formation of a mass of undifferentiated cells (blastema), which consists of archaeocytes, dedifferentiated choanocytes, anucleated amoebocytes, and differentiated spherulous cells. The main mechanisms of *A. cavernicola* regeneration are cell dedifferentiation with active migration and subsequent redifferentiation or transdifferentiation of polypotent cells through the mesenchymal-to-epithelial transformation. The main cell sources of the regeneration are archaeocytes and choanocytes. At early stages of the regeneration, the blastema almost devoid of cell proliferation, but after 24 hpo and up to 72 hpo numerous DNA-synthesizing cells appear there. In contrast to intact tissues, where vast majority of DNA-synthesizing cells are choanocytes, all EdU-labeled cells in the blastema are mesohyl cells. Intact tissues, distant from the wound, retains intact level of cell proliferation during whole regeneration process. For the first time, the apoptosis was studied during the regeneration of sponges. Two waves of apoptosis were detected during *A. cavernicola* regeneration: the first wave at 6-12 hpo and the second wave at 48-72 hpo.

Keywords: demosponges, regeneration, mesenchymal-to-epithelial transformation, blastema, apoptosis, transdifferentiation.

Highlights

- 1) Regeneration in the demosponge *Aplysina cavernicola* is accompanied with the formation of a mass of undifferentiated cells (blastema).
- 2) The main mechanisms of *A. cavernicola* regeneration are cell dedifferentiation with active migration and subsequent redifferentiation or transdifferentiation of polypotent cells - archaeocytes and choanocytes - through the mesenchymal-to-epithelial transformation.
- 3) Apoptosis during regeneration of *A. cavernicola* participate in damaged cells elimination and associated with the extensive ejection of spherulous cells from wound area.

64 Introduction

65 In spite of big interest in various problems concerning origin and early steps of
66 evolution in animal regeneration, morphogenesis, cell turnover etc., up to now there are
67 surprisingly small number of the studies, dealing with ultrastructural, morphogenetic, cell and
68 genetic aspects of sponge reparative regeneration.

69 Phylum Porifera consists of four classes: syncytial Hexactinellida, and cellular
70 Calcarea, Homoscleromorpha and Demospongiae. The last class is the largest and includes
71 about 80% of living sponges. Studies of regeneration in sponges have begun on demosponges
72 (Cavolini, 1785; Vaillant, 1869; Weltner, 1893). However, there are only few papers,
73 concerning ultrastructural description of the morphogenesis and cell behavior in reparative
74 regeneration of sponges. Moreover, three of them dealing with the regeneration of specific
75 “organs” after amputation (oscular diaphragm in *Hippospongia communis* (Thiney, 1972),
76 oscular tube in *Ephydatia fluviatilis* (Sukhodolskaya, 1973), and papillae in *Polymastia*
77 (Boury-Esnault, 1976)). Reparative regeneration of the body wall is described only in seven
78 species. Regeneration in *Spongilla lacustris* (Brondsted, 1953), *Halichondria panicea*
79 (Korotkova & Nikitin, 1969), *Geodia barretti* (Hofmann et al., 2003) and *Halisarca caerulea*
80 (Alexander et al., 2015) was studied only with light microscopy. In the case of *H. caerulea*,
81 the light microscopy studies were supplemented with the investigations of the cell
82 proliferation during the regenerative processes (Alexander et al., 2015). Reparative
83 regeneration in *Chondrosia reniformis* was investigated with light and scanning electron
84 microscopy (SEM) (Pozzolini et al., 2019). Finally, SEM studies supplemented with time-laps
85 recordings were done for regeneration in *Hymeniacidon heliophila* (Coutinho et al., 2017).

86 At the same time, our complex detailed investigations of reparative regeneration, done
87 with TEM, SEM, epifluorescent and light microscopy, immunocytochemistry and time-laps
88 recordings, in homoscleromorphs (Ereskovsky et al., 2015), calcareous sponges (Ereskovsky
89 et al., 2017; Lavrov et al., 2018) and demosponges (Borisenko et al., 2015) show a high
90 diversity of morphogenesis, cell mechanisms, and cell turnover, accompanying these
91 processes.

92 Thus, having comprehensive data about mechanisms of the regeneration for only one
93 species from the huge and very diverse class Demospongiae, we cannot make any
94 generalizations regarding the mechanisms of regeneration of this class of Porifera.

95 Representatives of the genus *Aplysina* are widely distributed in subtropical and tropical
96 coastal waters (Bergquist & Cook, 2002). They are considered proper models in chemical
97 ecology and microbiology (Azevedo et al., 2008; Betancourt-Lozano et al., 1998; Thoms et

1
2
3 98 al., 2004, 2006). *Aplysina cavernicola* is a popular and promising model for various
4
5 99 researches, dealing with the sponge cell composition (Vacelet, 1966, 1967, 1970, 1971, 1975;
6
7 100 Vacelet & Gallissian, 1978), bacterial symbionts (Vacelet, 1975; Fiedrich et al., 1999;
8
9 101 Hentschel et al., 2001; Thoms et al., 2003), three-dimensional skeletal scaffolds (Vacelet,
10
11 102 1971a,b; Garrobe et al. 1973; Ehrlich et al. 2010a, b), biochemistry and secondary metabolites
12
13 103 (D'Ambrosio et al., 1982, 1983; Ciminiello et al., 1997; Reverter et al., 2016), and temporal
14
15 104 variability of secondary metabolism (Reverter et al., 2016). Life cycle researches showed that
16
17 105 *A. cavernicola* is an oviparous sponge whose reproductive period lasts barely one month
18
19 106 (Gallissian & Vacelet, 1976; Reverter et al., 2016).

20
21 107 The present study was aimed at investigation of the reparative regeneration in
22
23 108 Mediterranean demosponge *Aplysina cavernicola* (Vacelet, 1959). The wide range of methods
24
25 109 allow us to make a comprehensive analysis of mechanisms, which contribute to the
26
27 110 regeneration in this species, including cell behavior and migrations, morphogenetic process,
28
29 111 cell proliferation and apoptosis.

30 112

31 113 **Materials and methods**

32 114

33 115 ***Sampling***

34 116 *Aplysina cavernicola* (Vacelet, 1959) (Demospongiae, Verongida) is a perennial
35
36 117 sciaphilous species inhabiting coralligenous formations or the entrance of submarine caves
37
38 118 generally between 8 to 60 m in the Mediterranean Sea (Figure 1). It presents a typical
39
40 119 yellowish color. For regeneration experiments *A. cavernicola* specimens were collected by
41
42 120 SCUBA diving in September-November 2017, July-August and October 2018 and March
43
44 121 2019 near Maire Island, Marseille (43.2096° N; 5.3353° E) at a depth of 12 - 15 m. Collected
45
46 122 sponges were maintained in a 100 l laboratory aquarium with running natural seawater at a
47
48 123 temperature of 15-16°C for 36 hours for sponge adaptation.

49 124

50 125 ***Surgical operations***

51 126 Two type of surgical operations have been conducted: i) *in situ*, and ii) in laboratory.
52
53 127 For *in situ* operations six individuals were used. The experiment was performed in June 2018
54
55 128 at the site of sponge sampling. In each sponge wounds were made in a wall of a cylindrical
56
57 129 outgrowth using a sharp stainless dissecting scalpel. The wounds had a uniform size of 3 cm²
58
59 130 in area and 1 cm deep. Each wound was measured and photographed at $t = 0, 2, 7, 12,$ and 32
60
61 131 days post operation, using digital camera Nikon D300 equipped with waterproof camera

1
2
3 132 housing SUBAL ND300 and flash INON Z-240.

4
5 133 Surgical operations in laboratory were performed as an excision of a small part
6
7 134 (approximately 0.3-0.5x0.3-0.5 cm) of the body wall at the base of a cylindrical outgrowth
8
9 135 (Figure 2). A total of 24 individuals were used in the body wall regeneration experiments
10
11 136 (Supporting Table 1).

12 137 The surgical operations were done manually under a stereomicroscope using scalpel.
13
14 138 After the operations the sponges with excised body wall were maintained in a 100 l laboratory
15
16 139 aquarium with running natural seawater at a temperature of 15-16°C. The sponges were
17
18 140 inspected and photographed using a stereomicroscope Leica M165FC (Leica) equipped with a
19
20 141 digital camera Leica DFC 320 (Leica) and LAS Store and Recall v.4.1 software (Leica). The
21
22 142 observations were done at 3, 6, 12, 18, 24, 36, 48, 72, 96, and 120 hours post operation (hpo).

23
24 143

24 144 ***Light and electron microscopy***

25 145 Specimens were fixed overnight at 4°C by 2.5% glutaraldehyde (Ted Pella) on 0.2M
26
27 146 cacodylate buffer (pH 7.4) and post-fixed for 2 h with 1% OsO₄ (Spi Supplies) on the same
28
29 147 buffer at room temperature (RT). Between fixation and post-fixation specimens were twice
30
31 148 rinsed with cacodylate buffer for 30 min. Finally, specimens were dehydrated in an ethanol
32
33 149 series at RT and stored in 70% ethanol at 4°C.

34 150 For semi-thin sections and transmission electron microscopy (TEM) specimens were
35
36 151 embedded in Araldite (Sigma-Aldrich) epoxy embedding media according to the
37
38 152 manufacturer instructions. Semi-thin sections (1 μm) were cut on a Reichert Jung
39
40 153 ultramicrotome (Reichert) and Ultramicrotome PowerTome XL (RMC Boeckeler) and then
41
42 154 stained with 1% toluidine blue – 0.2% methylene blue mixture. The semi-thin sections were
43
44 155 studied under a WILD M20 microscope (Wild). Digital photos were taken with a Leica
45
46 156 DMLB microscope (Leica) using Evolution LC color photo capture system
47
48 157 (MediaCybernetics).

48 158 Ultrathin sections (60–80 nm) were cut with a Leica UCT6 and an Ultramicrotome
49
50 159 PowerTome XL, equipped with a Drukkert 45° diamond knife, and contrasted with 4%
51
52 160 aqueous uranyl acetate. Ultrathin sections were studied under Zeiss-1000 (Carl Zeiss)
53
54 161 transmission electron microscope.

55 162 For scanning electron microscopy (SEM), fixed specimens were critical-point-dried,
56
57 163 sputter-coated with gold-palladium, and observed under Hitachi S 570 (Hitachi) microscope.

58
59 164

60 165 ***Spherulous cell counting***

1
2
3 166 Spherulous cells were counted on images of semi-thin sections of intact sponge tissues
4
5 167 and regenerating specimens at 6, 12, 24, 48, and 96 hpo. The images were obtained with a
6
7 168 Leica DMLB microscope (Leica) at 40x magnification using Evolution LC color photo
8
9 169 capture system (MediaCybernetics). At each stage three images, arising from three
10
11 170 independent individuals, were used for counting. Spherulous cells were counted in the
12
13 171 approximate 50- μ m thick lane beneath the exopinacoderm in intact sponges or beneath wound
14
15 172 surface in regenerating individuals. The area of the studied lanes was measured for each
16
17 173 image, and number of spherulous cells were extrapolated for an area of 1 mm². Cell counting
18
19 174 and area measuring were done with ImageJ v.1.48 software (National Institute of Health). For
20
21 175 each stage mean value and standard deviation were calculated (Supporting Table 4).

22 176 Statistical analysis was performed in R (R Core Team, 2019) with basic package “stats”
23
24 177 ver. 3.6.0 (R Core Team, 2019) and additional packages “agricolae” ver. 4.2-0 (de
25
26 178 Mendiburu, 2019), “car” 3.0-3 (Fox & Weisberg, 2019) and graphic package “ggplot2” ver.
27
28 179 3.3.1 (Wickham, 2019). To analyze the results, analysis of variance (ANOVA) was performed
29
30 180 to evaluate differences for spherulous cell count in intact tissues and at different stages of
31
32 181 regeneration. For ANOVA we prerequisitely performed box-cox transformation with $\lambda=0$ to
33
34 182 normalize our data, and Leven’s test for homogeneity of variances ($p = 0.3744$). For pairwise
35
36 183 comparisons, we performed Tukey’s honestly significant test and Duncan’s multiple range
37
38 184 test from “agricolae” package with 0.95 confidence level (Supporting Table 4).

39

37 185 38 186 ***Cell proliferation investigations***

39 187 A total of 27 individuals with the excised body wall were used in cell proliferation
40
41 188 studies (Supporting Table 2). The 5-Ethynyl-2'-deoxyuridine (EdU) (Thermo Fisher
42
43 189 Scientific), which incorporates in nuclear DNA during its synthesis in S-phase and marks
44
45 190 DNA-synthesizing cells, was used as a label for cell proliferation. The EdU stock solution
46
47 191 was prepared in DMSO (MP Biomedicals). The optimal EdU concentration and incubation
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49 192 time were elucidated during the preliminary studies with the intact tissues of *Aplysina*
50
51 193 *cavernicola*.

51 194 Labeling of DNA-synthesizing cells in the regenerating sponges were conducted during
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53 195 the following time periods: 0-6, 6-12, 0-24, 24-48, 48-72, 120-144 hpo. The EdU
54
55 196 concentration in the experiments with 6-hour incubation period was 600 μ M and in the
56
57 197 experiments with 24-hour incubation period – 200 μ M. Three individuals were used at each
58
59 198 time period. Three additional sponges were cultured in FSW without the EdU and served as
60
199 negative technical controls (Supporting Table 2).

1
2
3 200 The cell proliferation was also studied in the intact tissues of *A. cavernicola*. Three
4 201 individuals were incubated 6 hours in FSW with 600 μ M EdU and three individual – 24 hours
5 202 in FSW with 200 μ M EdU. One individual was cultured in FSW without the EdU and served
6 203 as a negative technical control (Supporting Table 2). The mode of EdU incubation did not
7 204 significantly influence the pattern of the staining of DNA-synthesizing cells: after both types
8 205 of incubation sponge tissues show essentially the same amount and localization of the DNA-
9 206 synthesizing cells.

15 207 During the EdU incubation, intact and regenerating sponges were cultivated in glass
16 208 vessels with 200 ml FSW supplemented with required amount EdU at 13°C.

18 209 After the incubation period, all individuals were rinsed twice with FSW and fixed with
20 210 4% PFA (Sigma-Aldrich) in PBS (Amresco, Inc.) for 12-15 hours at 4°C. Fixed specimens
21 211 were rinsed with PBS and the Click-iT reaction were performed in the following mixture: 4
22 212 mM CuSO₄ (ChimMed), 20 mg/ml Sodium L-ascorbate (Sigma-Aldrich) and 10 μ M Sulfo-
23 213 Cyanine3 Azide (Lumiprobe) in PBS. Finally, the specimens were rinsed several times with
24 214 PBS and stained with DAPI (Sigma-Aldrich).

29 215 Stained specimens were mounted in 90% glycerol-DABCO (Sigma-Aldrich) and
30 216 studied with a CLSM Nikon A1 (Nikon) using lasers with 405 nm, 488 nm and 546 nm
31 217 wavelength. The tissues beneath the wound surface and tissues no less than 1 cm away from
32 218 the wound were studied in each regenerating specimen.

36 219 The obtained Z-stacks and images were processed with ImageJ v.1.48 software
37 220 (National Institute of Health). Nuclei measuring were done on separate optical slices with NIS
38 221 Elements Viewer v. 4.5 (Nikon) and JR Screen Ruler v. 1.5 (Spadix Software). For all
39 222 measurements mean value and standard deviation were calculated.

43 223

44 224 ***Apoptosis investigation***

46 225 Four individuals were used for studies of apoptosis during regeneration and in intact
47 226 sponge tissues (Supporting Table 3). The studies were performed using the In Situ Cell Death
48 227 Detection Kit (Roche) or Click-iT Plus TUNEL Kit (Thermo Fischer Scientific). Both kits
49 228 detect apoptotic cells using the TUNEL assay, i.e. by attaching labeled nucleotides to double-
50 229 stranded DNA breaks that occur at the later stages of apoptosis.

55 230 Intact tissue and wounded areas at 6, 12, 24 and 48 hpo were fixed at 4°C by 4% PFA
56 231 (Sigma-Aldrich) on PBS (Amresco, Inc.). Fixed specimens were rinsed with PBS and treated
57 232 according to the manufacturer instructions for apoptotic cell visualization. Finally, the
58 233 specimens were rinsed several times with PBS and stained with DAPI (Sigma-Aldrich).

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3 234 Samples, incubated with DNase I recombinant purified from bovine pancreas (Thermo
4
5 235 Fisher Scientific) prior to the TUNEL reaction, were used as positive technical controls.
6
7 236 Samples, incubated without the TdT enzyme during the TUNEL reaction, were used as
8
9 237 negative technical controls.

10 238 Stained specimens were mounted in Mowiol (12%) or 90% glycerol-DABCO (Sigma-
11
12 239 Aldrich) and studied with a confocal microscope TCS-SPE (Leica) or CLSM Nikon A1
13
14 240 (Nikon) using lasers with 405 nm, 488 nm and 546 nm wavelength. The tissues beneath the
15
16 241 wound surface and tissues no less than 1 cm away from the wound were studied in each
17
18 242 regenerating specimen.

19 243

20 244 ***Field Study Permissions***

21
22 245 No specific permissions were required for these locations because the study was done
23
24 246 outside national parks, private lands or protected areas. We declare that the field studies did
25
26 247 not involve endangered or protected species.

27 248

28 29 249 **Results**

30 250

31 251 **Intact sponge morphology and histology**

32
33 252 The body of *Aplysina cavernicola* has a branchy shape, with each cylindrical branch
34
35 253 having 1-2 cm in diameter (Figure 1). Sponge tissues are dense and elastic. The sponge has
36
37 254 leuconoid organization of aquiferous system (numerous small choanocyte chambers, scattered
38
39 255 in the mesohyl). The skeleton represented exclusively by organic (spongin) fibers, covered
40
41 256 with chitin (Ehrlich et al., 2010a), thus the surgical operations are easily conducted.

42
43 257 The body is composed of the peripheral ectosome and the internal endosome, bearing
44
45 258 numerous choanocyte chambers (Figure 3A). The ectosomal region is up to 30 μm thick and
46
47 259 consists of three layers: (1) external parts of the T-shaped exopinacocytes, connected by non-
48
49 260 specialized cell junctions (Figure 3B,C) and covered by an acellular cuticle; (2) layer
50
51 261 containing collagen fibrils, cell bodies of exopinacocytes and scattered spherulous cells; and
52
53 262 (3) the inner layer, consisting of condensed collagen fibrils and spherulous cells. The
54
55 263 endosome (Figure 3A) composes the major part of the sponge body. It includes choanocyte
56
57 264 chambers, consisted of flagellated choanocytes, aquiferous canals, lined by endopinacocytes,
58
59 265 (Figure 3D,E) and the mesohyl with the skeleton, abundant symbiotic bacteria and scattered
60
61 266 specialized sponge cells.

1
2
3 267 Populations of free cells in the mesohyl of *A. cavernicola* include: lophocytes,
4
5 268 archaeocytes, pocket cells, contractile cells (myocytes) (Figure 3F,H), spherulous cells at
6
7 269 different stages of their maturation with two principal morphotypes: larger cells with clear
8
9 270 inclusions (Figures 3C, 4G) and smaller ones with dense inclusions (Figure 4D,H),
10
11 271 bacteriocytes, microgranular cells, spongocytes (Figure 4A-F) (Vacelet, 1966, 1967, 1970,
12
13 272 1971, 1975; Vacelet & Gallissian, 1978).

13 273

15 274 **Regeneration**

16
17 275 In spite of minor individual differences wound healing in *Aplysina cavernicola*, which
18
19 276 is expressed in the epithelialization of the wound surface, is completed within two – six days
20
21 277 (Figure 1). During our observations we did not reveal any significant differences in the onset
22
23 278 of the stages and course of the regeneration, as well as in the morphogenesis accompanying it,
24
25 279 across studied individuals. The regeneration ends within two weeks, when the wound is
26
27 280 completely healed: only a small depression on the surface remains in its place.

27 281 At the histological level the observed regeneration processes can be subdivided into
28
29 282 three stages: 1) internal milieu isolation – formation of a clot (3 – 12 hpo), 2) wound healing –
30
31 283 epithelization (12 – 24 hpo), and 3) restoration of ectosome and endosome (36 – 96 hpo).

32 284 For detailed description of morphogenesis and cell behavior, accompanying the
33
34 285 regeneration, we propose to subdivide a wound and tissue around the wound on several areas
35
36 286 (Figure 5):

- 37 287• Wound – a break in the continuity of any bodily tissue due to injury.
- 38 288• Wound area – tissues directly adjacent to an excised part of the sponge body; their structure is
39
40 289 severely disrupted during the surgery.
- 41 290• Edge of the wound – peripheral parts of the wound, which is in direct contact with intact
42
43 291 tissues.
- 44 292• Regeneration area – an area of the sponge body (tissue), which is not directly affected by
45
46 293 surgery, but in which anatomical structures (choanocyte chambers, canals of aquiferous
47
48 294 system and skeleton) are reorganized, and the normal composition and distribution of cells
49
50 295 disrupted due to their participation (dedifferentiation, transdifferentiation and migration) in
51
52 296 the regenerative processes. The dimension of this area could vary, depending on size and type
53
54 297 of the injury and on individual characteristics of a sponge.
- 55 298• Intact issues – ectosome and endosome areas that are not affected by surgery and are not
56
57 299 directly involved in regeneration and retaining the normal organization.

1
2
3 300 ***Stage I – Internal milieu isolation***

4
5 301 Immediately after the surgical excision of ectosome with the directly adjacent endosome,
6
7 302 the wound surface retracts, leaving the surface of the intact ectosome protruding around the
8
9 303 edges of the wound. The ectosome and the upper areas of endosome are destroyed in the
10
11 304 wound area.

12 305 During the first 3 hours post operation (hpo), the wound surface is covered with
13
14 306 exudate, cell debris and numerous symbiotic bacteria. The extracellular matrix (ECM) does
15
16 307 not show any signs of a condensation (Supporting Figure 1A-C). All cells in the wound area
17
18 308 undergo morphological modifications. The epithelial cells - endopinacocytes and choanocytes
19
20 309 of choanocyte chambers, begin losing contacts with adjacent cells in their epithelial layers and
21
22 310 change their shape from trapeziform (choanocytes) and flat (endopinacocytes) to spherical or
23
24 311 amoeboid (Figure 6). These dedifferentiated cells mix with the mesohyl cell population.
25
26 312 During transformation of the choanocytes, their collar of microvilli and flagellum are
27
28 313 resorbed (Figure 6D). However, the elements of the flagellar apparatus (the basal body and
29
30 314 accessory centriole located near the nucleus) persist in the transformed cells for
31
32 315 approximately two days and serve as the natural marker of dedifferentiated choanocytes. The
33
34 316 dedifferentiated endopinacocytes have no specific morphological characteristics and therefore
35
36 317 these cells cannot be distinguished from other mesohyl cells. The non-secreting cells of the
37
38 318 mesohyl (archaeocytes, lophocytes, dedifferentiated choanocytes and endopinacocytes)
39
40 319 actively phagocytosed cell debris, symbiotic and invasive microbes, including diatoms in the
41
42 320 wound area (Figure 6). Thus, all cells in the wound area, except the spherulous cells, are filled
43
44 321 with large phagosomes. The ectosome, surrounding the wound slightly contracts and bends
45
46 322 inward. After 3 hpo an active migration of the mesohyl cells and dedifferentiated choanocytes
47
48 323 towards the wound surface begins from wound area, as these cells assume an elongated shape
49
50 324 with the long axis, perpendicular to the wound surface (Figure 6). There are no changes in the
51
52 325 regeneration area in this period.

53
54 326 At 6 hpo, the wound surface is aligned and becomes flat and smooth. It is covered by a
55
56 327 thick layer of ECM, containing fragments of cells, dispersed symbiotic bacteria and few
57
58 328 spherulous cells (some of which are beginning their dedifferentiation) (Figure 7A; Supporting
59
60 329 Figure 1D). The amount of the spherulous cells in the wound area at this stage of the
330
331 regeneration significantly decreases and is approximately 2,8-fold lower than in the ectosome
332
333 of intact sponges (Figure 8; Supporting Table 4). This structure can be referred as a
regenerative clot, by analogy with other animals (Carlson, 2007). The thickness of the clot is
from 12 to 20 μm .

1
2
3 334 The cells of wound area have numerous small or few large phagosomes, which
4 335 contained fragments of the spherulous and granular cells (Figure 7B-F). Such cells could be
5 336 found not only in the upper part of the wound, but also in the deeper zone up to 100 μ m
6 337 beneath the wound surface. There still no visible changes in the regeneration area at this
7 338 stage.

8 339 At 12 hpo a regenerative clot at the wound surface is getting thinner, and wound surface
9 340 become aligned, flat and smooth (Figure 9A). The peripheral parts of the wound are clearly
10 341 limited by the flat outgrowths of intact exopinacocytes (Figure 9F).

11 342 In the wound area, cell distribution begins ordering, and ECM condensation occurs
12 343 (Supporting Figure 1E). The number of dedifferentiated choanocytes and endopinacocytes,
13 344 archaeocytes, and lophocytes increases in this area in comparison with the previous period of
14 345 regeneration (Figure 9B-E). Majority of these cells migrated from regenerated area. In
15 346 contrast, the number of the spherulous cells shows insignificant variations and remains
16 347 approximately the same as at 6 hpo (Figure 8; Supporting Table 4). Some of these spherulous
17 348 cells undergo the dedifferentiation, which is accompanied by the release of the spherules from
18 349 the cells. The amount of free mesohyl and concentration of the symbiotic bacteria decrease in
19 350 comparison with previous periods of regeneration (Figure 9C, D).

20 351 Simultaneously, the migration of the amoeboid cells (archaeocytes, dedifferentiated
21 352 choanocytes) from the regeneration area to the wound area and wound surface proceeds
22 353 (Figure 9C,E), and some of the migrating cells reach the wound surface, where they become
23 354 oriented with the long axis parallel to the wound surface (Figure 9B). Also first elongated
24 355 contractile cells (myocytes) appear in the regeneration area.

25 356

26 357 ***Stage II. Wound healing - epithelization (12 – 24 hpo)***

27 358 At 24 hpo a cell mass, consisting of heterogenous dedifferentiated and undifferentiated
28 359 (archaeocytes) cells, is formed under the upper part of the wound area. We considered it as a
29 360 blastema, as structurally it resembles blastemas formed during regeneration of other animals.
30 361 Blastema occupies the wound area and the upper part of the regeneration area.

31 362 Simultaneously, the upper part of the wound area becomes similar to intact sponge
32 363 ectosome, showing structured ECM with collagen fibrils (Figure 10A,B; Supporting Figure
33 364 1F). The wound surface is mosaically covered with very thin superficial outgrowths of
34 365 developing T-shaped exopinacocytes, arising from blastema cells of the heterogeneous origin
35 366 (Figure 10B,C,E). New exopinacocytes do not yet form close contacts with each other. In
36 367 some places submerged nucleated bodies of these cells are found. New exopinacocytes, in

1
2
3 368 contrast to the cells located deeper under the wound surface, have only few phagosomes.
4
5 369 There is no directed movement (contraction or creeping) of the intact exopinacoderm
6
7 370 surrounding the wound.

8 371 The number of spherulous cells in the wound area significantly increases in
9
10 372 approximately 2,3-folds in comparison with the previous stage of regeneration (Figure 8;
11
12 373 Supporting Table 4). These cells show typical appearance (Figure 10B). Their shape is oval,
13
14 374 not amoeboid, as in the earlier stages of regeneration. In contrast, near the wound surface, the
15
16 375 number of dedifferentiated choanocytes, archaeocytes and spherulous cells decreases (Figure
17
18 376 10C-F). The lophocytes, granular cells, and myocytes are absent from this zone. The
19
20 377 choanocyte chambers or their fragments completely disappear from the regeneration area at
21
22 378 this stage of regeneration. They occur at a depth of about 200 μm , as in intact sponge.

22 379

23 24 380 ***Stage III. Restoration of ectosome and endosome (36 – 96 h)***

25 381 At 48 hpo the ectosome of the regenerate is completely restored, but superficial cuticle,
26
27 382 characteristic for intact sponge, is still absent (Figure 11A,B; Supporting Figure 1G). The
28
29 383 wound epithelization is finished by new exopinacocytes, arose from the heterogeneous
30
31 384 dedifferentiated cells population and archaeocytes. Some of new exopinacocytes clearly show
32
33 385 their origin from the dedifferentiated choanocytes, as they still bear flagellar basal apparatus
34
35 386 (Figure 11C). According to the orientation of this flagellated complex, it can be assumed that
36
37 387 the basal part of the former choanocyte is flattened. Archaeocytes, reaching the wound, flatten
38
39 388 and assume position parallel to the surface (Figures 10E,F, 11D). New exopinacocytes have
40
41 389 normal T-shape and cell contacts.

41 390 During formation of the exopinacoderm active elimination of small apoptotic
42
43 391 spherulous cells with compact inclusions and cells, filled with big heterophagosomes, begins
44
45 392 (Figure 11E, F). However, the number of the spherulous cells shows insignificant variations
46
47 393 and remains approximately the same as at 24 hpo (Figure 8; Supporting Table 4).

48 394 During this stage, the choanocyte chambers and aquiferous canals of the endosome are
49
50 395 restored by association of previously disaggregated choanocytes and endopinacocytes,
51
52 396 respectively (Figure 12). Archaeocytes also participate in the development of new choanocyte
53
54 397 chambers (Figure 12D). Cells that form new structure of aquiferous system contact each other
55
56 398 and connect by interdigitations. Individual choanocytes form groups of cells, forming
57
58 399 structures less compact than in the intact choanocyte chamber (Figure 12D-F). These cells
59
60 400 gradually transform from mesenchymal morphology to epithelial.

1
2
3 401 At 96 hpo, the regeneration is almost complete: the ectosome obtains the cuticle, while
4 402 the endosome of the regeneration area begins to recover: choanocyte chambers and aquiferous
5 403 canals are gradually developing (Figure 13; Supporting Figure 1H). The number of
6 404 spherulous cells in the regenerated ectosome significantly increases, reaching the intact level
7 405 (Figure 8; Supporting Table 4). The free cells of the mesohyl, with the exception of
8 406 specialized secretory cells, still contain phagosomes.

13 407

15 408 ***Cell proliferation***

17 409 The DNA-synthesizing (EdU-labelled) cells are irregularly distributed in the intact
18 410 tissues of *A. cavernicola* (Figure 14). Numerous DNA-synthesizing cells are located in the
19 411 sponge endosome (Figure 14B). The majority of these cells are choanocytes with small
20 412 ($3.87 \pm 0.46 \mu\text{m}$; $n=45$) round to oval nuclei (Figure 14D). Nevertheless, some mesohyl cells
21 413 with large ($4.84 \pm 0.44 \mu\text{m}$; $n=50$) round nuclei are also labelled by EdU (Figure 14E). These
22 414 mesohyl DNA-synthesizing cells occurs all over sponge mesohyl: in the ectosome, endosome
23 415 and tissues, adjacent to the large exhalant canals of the aquiferous system, which are
24 416 structurally similar to the ectosome. The ectosome and tissues, adjacent to the large exhalant
25 417 canals contain only few such mesohyl DNA-synthesizing cells (Figure 14A, C).

27 418 During the regeneration, the pattern of the cell proliferation dramatically changes
28 419 (Figure 15). Immediately after the surgical operation, the level of the cell proliferation in the
29 420 tissues, adjacent to the wound, decreases sharply: at 0-6 hpo, DNA-synthesizing cells are
30 421 completely absent in a 100- μm zone below the wound surface (Figure 15A); at 6-12 hpo and
31 422 0-24 hpo, rare DNA-synthesizing cells appear in the tissues, located 30-40 μm below the
32 423 wound surface. The majority of the labelled cells are choanocytes from disintegrating
33 424 choanocyte chambers, however some mesohyl cells are also labelled. Similarly, to the intact
34 425 tissues, the labelled choanocytes have small ($3.85 \pm 0.4 \mu\text{m}$; $n=13$) round to oval nuclei, while
35 426 labelled mesohyl cells are characterized by large ($4.79 \pm 0.37 \mu\text{m}$; $n=16$) round nuclei.

36 427 After 24 hpo, further changes in the cell proliferation pattern occurs in the regeneration
37 428 area. The samples at 24-48 hpo and 48-72 hpo show a similar pattern (Figure 15B, D): in a
38 429 100- μm zone below the wound surface, numerous DNA-synthesizing cells occur. Some of
39 430 these cells are located just beneath the wound surface. Virtually all labeled cells are located in
40 431 the mesohyl, since the choanocyte chambers disappear in the tissues, adjacent to the wound,
41 432 by this time. These DNA-synthesizing cells have large ($4.99 \pm 0.39 \mu\text{m}$; $n=16$) round nuclei,
42 433 similar to EdU-labelled mesohyl cells from intact tissues. Occasionally, single DNA-

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3 434 synthesizing choanocytes occur in the tissues, located 60-70 μm below the wound surface,
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5 435 where intact choanocyte chambers could be retained.

6 436 At 120-144 hpo, after the recovery of ectosome and endosome structure in the
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8 437 regeneration area, the cell proliferation returns to the normal state, showing the intact pattern
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10 438 with majority of DNA-synthesizing cells, occurring in choanocyte chambers. However, the
11
12 439 level of cell proliferation is still lower than in intact tissues (Figure 15E).

13 440 In the tissues, distant from the wound, the intact cell proliferation pattern persists during
14
15 441 the whole regeneration process (Figure 15F).

16
17 442

18 443 *Apoptosis*

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20 444 We found no apoptotic cell in the intact tissue of *A. cavernicola*.

21
22 445 Two waves of apoptosis occur during the regeneration process. The level of apoptosis is
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24 446 low during both waves with few apoptotic cells, located only at the upper part of the wound.
25
26 447 In the endosome under the wound and in tissues distant from the zone of regeneration
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28 448 apoptotic cells are completely absent.

29 449 The first wave is associated with the early stages of regeneration. Apoptotic cells appear
30
31 450 at 6 hpo, and their number gradually decline during further regeneration (Figure 16A). At 12
32
33 451 hpo only single apoptotic cells occur at the upper parts of the wound area (Figure 16B), while
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35 452 at 24 hpo they are virtually absent. Apparently, this wave of apoptosis participates in the
36
37 453 elimination of damaged cells from the wound area.

38 454 The second wave of apoptosis occurs at 48 hpo, when relatively numerous apoptotic
39
40 455 cells appears at the upper parts of the wound area (Figure 16C). This wave is probably
41
42 456 associated with the active elimination of cells through the emerging exopinacoderm during
43
44 457 later stage of regeneration (Figure 11E,F).

45 458

46 459 **Discussion**

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48 460

49 461 **1 General**

50 462 *In vivo* observations

51 463 Sponges are well known for their capacity to regenerate not only small body parts, but
52
53 464 also after substantial partial mortality and damage. *In situ* monitoring of naturally and
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55 465 experimentally generated wounds confirms high recovery capacity of sponges from different
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57 466 taxa and of different growth forms (Connes, 1966, 1968; Ayling, 1983; Hoppe, 1988;
58
59 467 Duckworth, 2003; Henry & Hart, 2005; Wulff, 2010, 2013). These studies also discovered

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3 468 that regeneration can be influenced both by characteristics of the wound and by inherent
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5 469 characteristics of particular species of sponges.

6 470 Our observations *in situ* showed that wound epithelialization under natural conditions
7
8 471 occurs in *Aplysina cavernicola* during two days. Other investigations provided with different
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10 472 demosponges showed the same results (Maas, 1910; Brondsted, 1953; Connes, 1966, 1968;
11
12 473 Korotkova & Nikitin, 1969; Korotkova et al., 1983; Diaz, 1979; Hoppe, 1988; Hofmann et al.,
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14 474 2003; Wulff, 2010, 2013; Alexander et al., 2014; Borisenko et al., 2015; Pozzolini et al.,
15
16 475 2019).

17 476 Previous investigations of regeneration demonstrated that in many individuals of the
18
19 477 same species the amount of damage, type of damage, size of the sponge, and location on the
20
21 478 individual sponge can influence recovery, and even susceptibility to further damage by other
22
23 479 agents (Henry & Hart, 2005; Wulff, 2010, 2013). However, we find small individual
24
25 480 variability during regeneration of *A. cavernicola*, probably because the wounds were applied
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27 481 in the same areas of the sponges and had the same size.

28 482

29 483 **Histological observations**

30 484 In this work we showed that regeneration processes in *Aplysina cavernicola* in general
31
32 485 follow the stages similar to those described in other massive sponges with leuconoid
33
34 486 aquiferous system - Demospongiae and Homoscleromorpha (Brondsted, 1953; Korotkova &
35
36 487 Nikitin, 1969; Diaz, 1979; Hofmann et al., 2003; Borisenko et al., 2015; Ereskovsky et al.,
37
38 488 2015; Pozzolini et al., 2019), and in various eumetazoans (Korotkova, 1997; Carlson, 2003).
39
40 489 Therefore, *A. cavernicola* regeneration includes three main stages: 1) internal milieu isolation
41
42 490 - formation of “regenerative clot”, 2) wound healing - epithelization, and 3) restoration of
43
44 491 damaged structures - ectosome and endosome. The wound epithelization in *A. cavernicola*
45
46 492 occurs without formation of the regenerative membrane, a structure, characteristic for
47
48 493 regeneration of asconoid and syconoid calcareous sponges (Jones, 1957; Korotkova, 1961,
49
50 494 1962; Ereskovsky et al., 2017; Lavrov et al., 2018).

51 495 The main mechanisms of *Aplysina cavernicola* reparative regeneration of the body wall
52
53 496 are (1) cell dedifferentiation with their subsequent redifferentiation, (2) transdifferentiation,
54
55 497 and (3) active migration of polypotent cells (archaeocytes and choanocytes) to the wound.
56
57 498 The same basic mechanisms are also characteristic for regeneration in other studied
58
59 499 demosponges: *Halichondria panicea* (Korotkova & Nikitin, 1969), *Suberites massa* (Diaz,
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500 1979), *Halisarca dujardini* (Borisenko et al., 2015), and *Suberites domuncula* (our
501 unpublished results). However, these mechanisms strongly differ from those, participating in

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2
3 502 regeneration process in calcareous sponges and homoscleromorphs, which show clear
4 503 epithelial organization with specialized cell junctions in the epithelia (Ereskovsky et al., 2015;
5 504 Lavrov et al., 2018).

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8 505

10 506 **2. Morphogenetic mechanisms of *A. cavernicola* regeneration**

11 507 Main morphogenesis during body wall regeneration in *A. cavernicola* is mesenchymal-
12 508 to-epithelial transformation (MET). Indeed, at 12 hpo archaeocytes and dedifferentiated
13 509 choanocytes as well as the spherulous cells begin to move toward wound area, where they
14 510 form a blastema-like structure. Part of external blastema cells begins to flat and form cover
15 511 sponge epithelium (exopinacoderm) through the MET.

16 512 However, experimentally induced (by surgical operation) epithelial-to-mesenchymal
17 513 transition (EMT) proceeds MET in *A. cavernicola*. During this process choanocytes and
18 514 endopinacocytes leave the choanocyte chambers and the canals of aquiferous system,
19 515 correspondingly, and move into the mesohyl, dedifferentiating and assuming an amoeboid
20 516 shape. This morphogenesis is one of central for the creation of numerous organs and complex
21 517 tissues during embryonic development, asexual reproduction, and regeneration and has been
22 518 well described in Eumetazoa (Keller et al., 2003; Lim & Thiery, 2012). EMT is also well
23 519 known during the normal ontogeny of sponges and occurs in the course of embryonic
24 520 development, and larval metamorphosis (reviewed in Ereskovsky et al. 2013). This
25 521 morphogenesis was described during the first stages of regeneration of other investigated
26 522 demosponges and homoscleromorphs (Korotkova & Nikitin, 1969; Borisenko et al., 2015;
27 523 Ereskovsky et al., 2015).

28 524 After wound epithelization, the restoration of elements of the aquiferous system begins.
29 525 Both choanocyte chambers and aquiferous system canals are formed through MET: separate
30 526 amoeboid cells coalesce into groups and gradually transform from mesenchymal
31 527 morphology to epithelial-like cells (choanocytes and endopinacocytes, respectively).

32 528 The same MET mechanism is characteristic for exopinacoderm and choanoderm
33 529 formation during regeneration of other investigated demosponges: *Halichondria panicea*
34 530 (Korotkova & Nikitin, 1969), *Halisarca dujardini* (Borisenko et al., 2015), and
35 531 basopinacoderm formation in *Hymeniacidon heliophila* regeneration (Coutinho et al., 2017).

36 532 It is fundamentally different from the regeneration in Calcarea and Homoscleromorpha,
37 533 in which epithelial morphogenesis, e.g. flattening and spreading of epithelial layers, plays a
38 534 leading role in the restoration of main structures (exopinacoderm and elements of aquiferous
39 535 system) (Ereskovsky et al., 2015; Lavrov et al., 2018).

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3 5364
5 537 **3. Blastema**

6 538 In regenerative biology blastema is a mass of undifferentiated cells, located beneath the
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8 539 wound and produced by the dedifferentiation of many cell types and/or migration of
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10 540 polypotent cells. In some cases, differentiation of blastema cells, and further reconstruction
11
12 541 the lost part of the body could be accompanied with cell proliferation. In this case, the mitotic
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14 542 rate of the blastema slows down as the structure grows, and it ceases completely when the
15
16 543 new structure reaches the original size (Santos-Ruiz et al., 2002; Carlson, 2007; Tsonis, 2008;
17 544 Vervoort, 2011).

18
19 545 The formation of a concentration of cells under the wound in the middle stages of a
20
21 546 body wall regeneration has been shown for several demosponges (Thiney, 1972; Boury-
22 547 Esnault, 1976; Borisenko et al., 2015; Coutinho et al., 2017). This cellular concentration
23
24 548 consists of archaeocytes and choanocytes, which are principal stem cells of demosponges
25
26 549 (Funayama, 2018), dedifferentiated cells (e.g., pinacocytes) and specialized differentiated
27 550 cells (e.g., gray cells) (Thiney, 1972; Boury-Esnault, 1976; Borisenko et al., 2015; Coutinho
28
29 551 et al., 2017). This structure could be referred as a blastema, characteristic for a regeneration in
30
31 552 many animals.

32
33 553 Similarly, in the current study, we refer to the undifferentiated cell mass beneath a
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35 554 wound during the end of second and beginning of third stages of regeneration in *A.*
36 555 *cavernicola* as a blastema. In *A. cavernicola*, it consists of archaeocytes, dedifferentiated
37
38 556 choanocytes, mixed population of anucleated amoebocytes (dedifferentiated pinacocytes,
39
40 557 myocytes, lophocytes), and differentiated spherulous cells.

41 558 At early stages of regeneration, the blastema almost devoid of DNA-synthesizing cell,
42
43 559 but after 24 hpo and up to 72 hpo numerous DNA-synthesizing cells appears there. However,
44
45 560 even during this period the level of cell proliferation in the blastema is much lower in
46
47 561 comparison with intact sponge tissues. In contrast, intact tissues distant from the wound
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49 562 retains intact level of cell proliferation during whole regeneration process. The similar pattern
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51 563 of cell proliferation has been described during body wall regeneration of *Halisarca caerulea*
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53 564 (Alexander et al., 2014) and *Halisarca dujardini* (Borisenko et al., 2015), for which
54
55 565 decreased proliferation in the wound area is characteristic.

56 566 Moreover, in contrast to intact tissues of *A. cavernicola*, where vast majority of DNA-
57
58 567 synthesizing cells are choanocytes, all EdU-labeled cells in the blastema are mesohyl cells, as
59
60 568 blastema lacks choanocyte chambers. These EdU-labeled cells could arise directly in blastema
569 through cell divisions or be EdU-labeled migrating descendants of proliferating cells in intact

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3 570 tissues, distant from the wound. This issue requires further investigations, using additional
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5 571 markers, like antibodies against phospho-histone H3 for revealing cells in M-phase and cell
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7 572 tracers for the visualization of cell migrations.

8 573 Importantly, studied sponges from classes Calcarea (*Leucosolenia cf. variabilis*)
9
10 574 (Lavrov et al., 2018) and Homoscleromorpha (*Oscarella lobularis*) (Ereskovsky et al., 2015)
11
12 575 demonstrate a distinct mode of regeneration without blastema formation. In these sponges
13
14 576 regeneration occurs due to the local remodeling of intact tissues, adjacent to the wound.
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16 577 Moreover, the cell proliferation is neither affected nor contributes to the regeneration at any
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18 578 stage.

19 579

20 580 **4. Dedifferentiation, transdifferentiation and cell sources in *A. cavernicola***
21
22 581 **regeneration**

23
24 582 Transdifferentiation is the transformation of one type of already differentiated cell into
25
26 583 another type of differentiated cell. In some cases, transdifferentiation is accompanied by cell
27
28 584 division, while in other cases it is not (Shen et al., 2004).

29 585 Sponge cell transdifferentiation is likely a driving force accompanying their restoration
30
31 586 processes (Korotkova, 1997; Lavrov & Kosevich, 2014; Adamska, 2018). However,
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33 587 transdifferentiating cell types and mechanisms of this process vary in different sponge species
34
35 588 (Diaz, 1979; Gaino et al., 1995; Korotkova, 1997; Ereskovsky et al., 2015; Borisenko et al.,
36
37 589 2015; Lavrov & Kosevich, 2016; Lavrov et al., 2018). For example, direct transdifferentiation
38
39 590 of cells in intact epithelia (pinacocytes and choanocytes) has been demonstrated during the
40
41 591 reparative regeneration of the homoscleromorph *Oscarella lobularis* (Ereskovsky et al.,
42
43 592 2015), the calcareous sponges *Sycon lingua* (Korotkova, Efremova, & Kadantseva, 1965;
44
45 593 Korotkova, 1972b), and *Leucosolenia* ssp. (Lavrov et al., 2018). In these cases, the layer of
46
47 594 choanocytes transdifferentiates into layer of pinacocytes without loss of epithelial structure,
48
49 595 without mesenchymal-to-epithelial transformation, and without contribution from cell
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51 596 proliferation.

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53 597 Cell transdifferentiation have also been described during reparative regeneration in all
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55 598 investigated demosponges (for review see: Korotkova, 1997; Borisenko et al., 2015). While
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57 599 archaeocytes directly differentiate into new cells, choanocytes and pinacocytes undergo
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61 600 transdifferentiation to give rise for other cell types. It is characteristic, mainly, for
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63 601 choanocytes from disintegrated choanocyte chambers, which can transdifferentiate, for
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65 602 example, into exopinacocytes (Korotkova & Nikitin, 1969a,b; Borisenko et al., 2015).
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67 603 Endopinacocytes in a less extent could also transdifferentiate into exopinacocytes (Thiney,

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2
3 604 1972; Borisenko et al., 2015). The principal difference of the transdifferentiation in
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5 605 demosponges in comparison with Homoscleromorpha + Calcarea lineage is an occurrence of
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7 606 the disruption of epithelial layers and cell dedifferentiation prior to this process in the
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9 607 demosponges.

10 608 The similar processes were observed during *A. cavernicola* regeneration. The
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12 609 archaeocytes and dedifferentiated cells participate in the restoration of the lost structures in
13
14 610 this sponge. We identify two cells types, which undergo dedifferentiation during *A.*
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16 611 *cavernicola* regeneration: choanocytes, spherulous cells, and population of anucleated cells of
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18 612 mesohyl, including dedifferentiated pinacocytes, myocytes, and lophocytes. The choanocytes,
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20 613 despite their dedifferentiation and loss of the flagella and collars of microvilles, are well
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22 614 distinguished on ultrathin sections due to the persistence of two centrioles in their apical part.
23
24 615 Anucleated amoebocytes differ from archaeocytes by a smaller nucleus without nucleolus and
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26 616 the presence of small phagosomes. The dedifferentiation of the spherulous cells through a
27
28 617 complete or almost complete loss of specific inclusions (spherules) was completely
29
30 618 unexpected process. However, the dedifferentiation of spherulous cells is not so rapid, as in
31
32 619 other dedifferentiating cells, because of high specialization of these secretory cells.

33 620 We identified two main cellular sources for the restoration of the lost tissue and
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35 621 structures in *A. cavernicola*: archaeocytes and choanocytes. Both cell types contribute to the
36
37 622 restoration of the exopinacoderm and choanocyte chambers. The restoration of the choanocyte
38
39 623 chambers is carried out mainly due to the redifferentiation of the choanocytes, and to lesser
40
41 624 extent due to the differentiation of archaeocytes.

42 625 However, the future of dedifferentiated anucleated amoebocytes and spherulous cells
43
44 626 could not be traced. This issue requires the search and use of a special marker of these cells. It
45
46 627 is possible that dedifferentiated pinacocytes and spherulous cells could also participate in the
47
48 628 formation of the new exopinacoderm.

49 629 According to last investigations in Porifera there are not only two types of adult stem
50
51 630 cells (ASC): archaeocytes and choanocytes (Funayama, 2018), but at least four, including
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53 631 pinacocytes and particular amoeboid vacuolar cells (Ereskovsky et al., 2015; Fierro-Constain
54
55 632 et al., 2017; Lavrov et al., 2018).

56 633 There are the differences in these ASC distributions among different sponge classes. In
57
58 634 Demospongiae the principal (pluripotent) ASC are archaeocytes and choanocytes, however
59
60 635 the experiments with their regeneration (including present data on *A. cavernicola*
636 regeneration) and dissociated cells aggregation demonstrated, that pinacocytes could also be
637 ASC (Korotkova & Nikitin, 1969a,b; Borisenko et al., 2015; Lavrov & Kosevich, 2016;

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2
3 638 Ereskovsky et al., 2016). In *Calcarea* archaeocytes absent and pluripotent ASC in these
4
5 639 sponges are choanocytes and pinacocytes (Korotkova, 1961a, 1962b; Korotkova &
6
7 640 Gelihovskaia, 1963; Lavrov et al., 2018). In Homoscleromorpha the pluripotent ASC are as
8
9 641 in *Calcarea* the choanocytes and pinacocytes, but also mesohyl amoeboid vacuolar cells
10 642 (Gaino et al., 1986; Ereskovsky, 2010; Ereskovsky et al., 2015; Fierro-Constain et al., 2017).

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12 643

13 644 **5. Spherulous cells and their role in regeneration**

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15 645 Similarly, to intact tissues of *A. cavernicola* (Vacelet, 1967), two principal morphotypes
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17 646 of spherulous cells occurs in the wound area of this sponge: larger cells with clear inclusions
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19 647 and smaller ones with dense inclusions. In fact, these morphotypes represent different stages
20
21 648 of the ontogenesis of spherulous cells (Vacelet, 1967). According to Vacelet (1967) in intact
22
23 649 sponge “dense” small spherulous cells concentrate mostly in the ectosome and around big
24
25 650 exhalant canals, while “clear” larger ones are distributed in the mesohyl of endosome.

26 651 We have detected that in *A. cavernicola* the wound surface was free from invasive
27
28 652 microbes from the first hours after injury up to the end of the regeneration. All microbes, that
29
30 653 we detected at the wound surface and in the wound area during regeneration, shows
31
32 654 ultrastructure identical to the microbes, located in the deeper sponge tissues and described as
33
34 655 symbiotic in the previous papers (Vacelet, 1975; Fiedrich et al., 1999; Hentschel et al., 2001).
35
36 656 Such defense against invasive microbes is probably provided by the particular chemical
37
38 657 substances in the spherulous cells.

39 658 X-ray microanalysis revealed that these specialized cells of *Aplysina* produce and store
40
41 659 brominated metabolites (Turon et al., 2000). Turon et al. (2000) demonstrated that the
42
43 660 concentration of bromine peaks is slightly higher in the spherules from “dense” spherulous
44
45 661 cells than in “clear” ones. Thompson et al. (1983) have detected bromine in the spherulous
46
47 662 cells of Caribbean *Aplysina fistularis*. Spherulous cells have been shown to produce defense
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49 663 metabolites in other species (Thompson et al., 1983; Bretting et al., 1983; Uriz et al., 1996a).

50 664 In addition, it was showed that tissue damage in *Aplysina* induces a bioconversion of
51
52 665 isoxazoline alkaloids into aeroplysinin-1 and secondary metabolite dienone (Thoms et al.,
53
54 666 2006). This reaction is likely catalyzed by enzymes, and it may be ecologically important as
55
56 667 the dienone has the strong antibacterial power and toxicity (Reverter et al., 2016). Moreover,
57
58 668 this injury-induced reaction takes place within less than 1 min after wounding (Thoms et al.,
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60 669 2006).

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61 671 **6. Apoptosis during *A. cavernicola* regeneration**

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3 672 We have directly visualized and investigated apoptosis in the regeneration of sponges
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5 673 for the first time. The only previous data on the involvement of apoptosis in sponge
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7 674 regeneration emerge from the comparative transcriptomic analysis of the early stages of body
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9 675 wall regeneration in *Halisarca caerulea* (Kenny et al., 2017).

10 676 We have observed two waves of apoptosis during *Aplysina cavernicola* regeneration.
11
12 677 The first wave, occurring around 6 hpo, is probably involved in the elimination of damaged
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14 678 cells and is characteristic for the early stages of regeneration of various animals, from
15
16 679 cnidarians to vertebrates (Vlaskalin et al., 2004; Tseng et al., 2007; Chera et al., 2009;
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18 680 Pellettiere et al., 2010; DuBuc et al., 2014; Brandshaw et al., 2015; Kenny et al., 2017; Cebrià
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20 681 et al., 2018). For *Hydra* head regeneration after mid-gastric bisection (Chera et al., 2009) and
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22 682 tail regeneration in tadpole larva of *Xenopus laevis* (Tseng et al., 2007) it was shown that
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24 683 early wave of apoptosis not only clean a wound from damaged cells, but also generate signals,
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26 684 essential for initiation of subsequent regenerative processes. Inhibition of this apoptotic wave
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28 685 completely abolishes the regeneration. In particular, in both cases, apoptotic cells initiate a
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30 686 synchronous burst of proliferative activity in neighboring cells (Tseng et al., 2007; Chera et
31
32 687 al., 2009), which is similar to the well-known apoptosis-induced compensatory proliferation
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34 688 in the imaginal disks of *Drosophila* (Fan & Bergmann, 2008; Bergmann & Steller, 2010).
35
36 689 Apoptotic cells may be involved in the proliferation induction also during *A. cavernicola*
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38 690 body wall regeneration, as numerous DNA-synthesizing cells appears in the blastema only
39
40 691 after the first apoptotic wave, at 24 hpo. These observations illustrate a novel active
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42 692 instructing role of apoptosis in morphogenetic processes, in contrast to its canonical passive
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44 693 role as a destructive agent (Duffy, 2012).

41 694 The second wave of apoptosis during *A. cavernicola* regeneration is likely associated
42
43 695 with the extensive thrown out of spherulous cells though the forming exopinacoderm.
44
45 696 Spherulous cells of *A. cavernicola* demonstrate the similar behavior in the intact sponges.
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47 697 Being one of the few terminally differentiated sponge cell lines, spherulous cells leave the
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49 698 mesohyl through aquiferous system canals or external surfaces (Vacelet, 1967). Their release
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51 699 outside from sponge is part of their normal physiology and possibly involved in the release of
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53 700 various chemicals (Uriz et al., 1996a; Ternon et al., 2016) and/or discharge processes
54
55 701 (Maldonado, 2016) to the environment. The ultrastructure of spherulous cells eliminated
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57 702 through the exopinacoderm during the third phase of *A. cavernicola* regeneration is identical
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59 703 to that of the spherulous cells at the last stage of their evolution (Vacelet, 1967). Similarly, in
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61 704 the intact tissues of *H. caerulea*, many caspase-3 positive cells have a spherulous cell
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63 705 morphology (De Goeij et al., 2009), which indicates that apoptosis involved in the waste

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3 706 control system in sponges in addition to maintaining tissue homeostasis (De Goeij et al.,
4 707 2009).

5
6 708 Thus, the described increase in the level of apoptosis at the late stages of the *A.*
7 709 *cavernicola* regeneration could be referred as a general tissue response, involved in the
8 710 restoration of the normal sponge physiology in the area of injury, rather than in regeneration
9 711 process itself. In planarians, the similar late general apoptotic response is also described, but it
10 712 participates in the intensive remodeling of intact tissues, adjacent to a wound, to restore
11 713 proper scale and proportions (Pellettieri et al., 2010; Cebrià et al., 2018).
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18 715 **Conclusion**

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20 716 Finally, we can made some principal conclusions:

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22 717 1. In Porifera there are two principal mode of reparative regeneration: blastemal
23 718 regeneration and tissue remodeling. From the literature data and results, obtained in this work
24 719 we can conclude that first mode is characteristic for Demospongiae regeneration and the
25 720 second – for Calcarea + Homoscleromorpha clade.
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29 721 2. The results of our research also showed that sponges have more than two lines of
30 722 adult stem cells, the potencies of which are clearly manifested during reparative regeneration.
31 723 Our data support previous investigations, showed that archaeocytes and choanocytes are main
32 724 players, or principal adult stem cells during demosponges regeneration (Korotkova & Nikitin,
33 725 1969; Thiney, 1972; Boury-Esnault, 1976; Diaz, 1979; Borisenko et al. 2015). However, the
34 726 pinacocytes also could be considered as multipotent stem cells. Moreover, for the first time
35 727 we showed, that spherulous cells of *Aplysina cavernicola* have a capacity to dedifferentiation
36 728 during regeneration. It is first evidence that highly specialized cells of demosponges have
37 729 capacity to dedifferentiation and possibly transdifferentiation.
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44 730 3. Cell transdifferentiation plays an extremely significant role in sponge regeneration, as
45 731 we have shown that regardless of the phylogenetic position, type of aquiferous system, and
46 732 structure of epithelia, all studied sponges intensively utilize it for restoration of lost structures
47 733 (Borisenko et al., 2015; Ereskovsky et al., 2015, 2017; Lavrov et al., 2018).
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51 734 4. For the first time was investigated apoptosis during sponge regeneration. In
52 735 *Aplysina cavernicola* regeneration this processes participate in damaged cells elimination and
53 736 associated with the extensive ejection of spherulous cells from wound area.
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58 738 **Author contributions**

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3 739 A.V.E. and A.I.L. designed the study, conducted the experimental procedures with the living
4 740 animals; A.I.L. performed the cell proliferation studies; A.V.E. and D.B.T. performed
5 741 histological and ultrastructural studies. A.V.E.: Project administration and funding
6 742 acquisition; S.B., E.L.G., and A.I.L.: performed the apoptosis studies; A.V.E. and A.I.L.
7 743 prepared the manuscript with contributions from all authors. All authors reviewed and
8 744 approved the final manuscript.
9 745

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3 1020 **Figure legends for *Aplysina cavernicola* regeneration**

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6 1022 **Figure 1.** Time series photographs *in situ* of wounds to *Aplysina cavernicola*.

8 1023 Time series photographs of wounds to *Aplysina cavernicola*, Corrals Cave, Marseille, France
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10 1024 June-July 2018. Two examples were chosen to illustrate individual variety of regeneration
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12 1025 after experimental wounding. W – wound. Scale bar – 1.2 cm.

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15 1027 **Figure 2.** *Aplysina cavernicola in vivo*

17 1028 A – intact sponge before operation; B – sponge just after operation; C – sponge after 48 hours
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19 1029 after operation.

20 1030 o – osculum, w – wound.

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24 1032 **Figure 3.** Intact *Aplysina cavernicola*

25 1033 A – semi-fin section of upper part of sponge; B – TEM image of ectosome; C –
26 1034 exopinacocyte and spherulous cells (inset: detail of cell junctions between exopinacocytes); D
27 1035 – endopinacocyte; E – choanocyte chamber (inset: choanocyte); F – pocket cell with
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29 1036 symbiotic bacteria; G – archaeocyte; H – myocytes (inset: detail of myocytes with
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31 1037 myofibrils).

34 1038 ar – archaeocyte, cb – cell body of exopinacocyte, cc – choanocyte chamber, ch – choanocyte,
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36 1039 ect – ectosome, en – endopinacocyte, end – endosome, ex- exopinacocytes, f – flagellum, fp –
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38 1040 flat part of exopinacocyte, in – inclusions in spherulous cells, my – myocyte, pc - pocket cell,
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40 1041 n – nucleus, sb – symbiotic bacteria, sc – spherulous cell.

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43 1043 **Figure 4.** Cells of the mesohyl in intact *Aplysina cavernicola*

44 1044 A – bacteriocyte; B – spongocyte; C – lophocytes; D - spherulous cell at the late stage of
45
46 1045 evolution expelled into exhalant canal; E – microgranular cell; F – granular cell; G, H –
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48 1046 spherulous cells in two stages of their development.

49 1047 ar – archaeocyte, ba – bacteriocyte, ex- exopinacocytes, exc – exhalant canal, in – inclusions
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51 1048 in spherulous cells, lo – lophocyte, mc – microgranular cell, n – nucleus, sb – symbiotic
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53 1049 bacteria, sc – spherulous cell, sf – skeleton fiber, sp - spongocyte.

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56 1051 **Figure 5.** Scheme of wound and their parts in *Aplysina cavernicola*.

58 1052 ect – ectosome, end – endosome, it - intact tissues, ra – regenerated area, w – wound, wa –
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60 1053 wound area, we – wound edge.

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Figure 6. 3h of regeneration in *Aplysina cavernicola*

1056 A – wound and a marginal zone; B – zone under the wound; C – internal part of the wound
1057 with encapsulated invasive diatoms; D – dedifferentiated choanocyte (flash show the basal
1058 flagellum apparatus)
1059 ar – archaeocyte, ba – bacteriocyte, dch – dedifferentiated choanocyte, di – encapsulated
1060 diatom, en – endopinacocyte, in – inclusions in spherulous cells, lo – lophocyte, n – nucleus,
1061 ph – phagosome, sb – symbiotic bacteria, sc – spherulous cell, scf – spherulous cells
1062 fragments, w - wound.

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Figure 7. 6h of regeneration in *Aplysina cavernicola*

1065 A – semi thin section of the wound and upper part of endosome; B, C – TEM of the upper
1066 part of the wound: it is a choanocyte chamber during their destruction, fragments of
1067 spherulous cells, phagocyted spherulous cell; D, E – wound zone with the fragments of cells
1068 and phagocytosis of spherulous cells; F – inner part of the wound, degraded choanocyte
1069 chamber.
1070 ar – archaeocyte, cc – choanocyte chamber, dcc - destructed choanocyte chamber, dch –
1071 dedifferentiated choanocyte, end – endosome, in – inclusions in spherulous cells, n – nucleus,
1072 ph – phagosome, sb – symbiotic bacteria, sc – spherulous cell, scf – spherulous cells
1073 fragments, w – wound, ws – wound surface.

1074

1075 **Figure 8.** Boxplot diagram of the number of spherulous cells per mm² in intact tissues and
1076 during regeneration in *A. cavernicola*. The differences between some of the means are
1077 statistically significant (ANOVA p-value = 2.36E-07(<0.01)). Tukey's multiple comparisons
1078 showed no significant differences only in 3 pairs: Intact tissues–96 hpo; 6 hpo–12 hpo; 24
1079 hpo–48 hpo. Duncan's new multiple range test (MRT) approve this, grouping these pairs into
1080 three clusters (A, B, C). The differences between Duncan's clusters are statistically significant
1081 (Supporting Table 4).

1082

Figure 9. 12h of regeneration in *Aplysina cavernicola*

1084 A – semi thin section of the wound and upper part of endosome; B, C – TEM of the upper
1085 part of the wound, there are some non-secret cells at the wound surface (arrowheads),
1086 dedifferentiated choanocytes and spherulous cells; D – detail of the upper part of the wound;

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3 1087 E – wound zone with dedifferentiated choanocytes; F – marginal zone of the wound with the
4 1088 part of intact exopinacocyte.

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6 1089 ar – archaeocyte, cf – cells fragments, dch – dedifferentiated choanocyte, dsc -
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8 1090 dedifferentiated spherulous cell, end – endosome, in – inclusions in spherulous cells, n –
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10 1091 nucleus, ph – phagosome, sb – symbiotic bacteria, sc – spherulous cell, scf – spherulous cells
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12 1092 fragments, w – wound, ws – wound surface.

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15 1094 **Figure 10. 24h of regeneration in *Aplysina cavernicola***

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17 1095 A – semi thin section of the wound and upper part of endosome; B, C – TEM of the upper
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19 1096 part of the wound, with flat parts of new exopinacocytes, spherulous and non-secret cells with
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21 1097 the phagosomes (arrowhead - basal body of choanocyte flagellum); D – wound zone with
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23 1098 dedifferentiated choanocytes; E - upper part of the wound zone with archaeocyte and
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25 1099 dedifferentiated choanocyte in the beginning of differentiation into new exopinacocyte, arrow
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27 1100 show the basal apparatus of dedifferentiated choanocyte; F - archaeocyte differentiated into
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29 1101 exopinacocyte at the upper part of the wound.

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31 1102 ar – archaeocyte, cf – cells fragments, dch – dedifferentiated choanocyte, dsc -
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33 1103 dedifferentiated spherulous cell, end – endosome, in – inclusions in spherulous cells, n –
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35 1104 nucleus, nu – nucleolus, ph – phagosome, sb – symbiotic bacteria, sc – spherulous cell, scf –
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37 1105 spherulous cells fragments, w - wound.

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38 1107 **Figure 11. 48h of regeneration in *Aplysina cavernicola***

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40 1108 A – semi thin section of the wound and upper part of endosome; B – TEM of the upper part of
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42 1109 the wound, with restored ectosome; C – TEM of the upper part of the wound, showed
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44 1110 transdifferentiation of a choanocyte into exopinacocyte; inset – basal apparatus of
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46 1111 dedifferentiated choanocyte (arrowhead); D – TEM of the upper part of the wound, showing
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48 1112 transdifferentiation of an archaeocyte into exopinacocyte; E, F - TEM of the small apoptotic
49
50 1113 spherulous cells elimination of apoptotic spherulous cells through forming exopinacoderm.

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52 1114 ac – apoptic cell, asc – apoptic spherulous cell, ar – archaeocyte, cc – choanocyte chamber, ch
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54 1115 – choanocyte, cf – cells fragments, dch – dedifferentiated choanocyte, end – endosome, ex-
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56 1116 exopinacocytes, fp – flat part of exopinacocyte, in – inclusions in spherulous cells, lo –
57
58 1117 lophocyte, mc – microgranular cell, my – myocyte, n – nucleus, ph – phagosome, sb –
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60 1118 symbiotic bacteria, sc – spherulous cell, scf – spherulous cells fragments, w – wound zone.

58 1119

60 1120 **Figure 12. 48h of endosome regeneration in *Aplysina cavernicola***

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3 1121 A-C – TEM of the endosome under the wound, showing different stages of restoration of
 4 1122 exhalant canals of the aquiferous system; D-F - TEM of the endosome under the wound,
 5 1123 showing different stages of restoration of choanocyte chambers.

6 1124 cc – choanocyte chamber, ch – choanocyte, en – endopinacocyte, exc – exhalant canal, sb –
 7 1125 symbiotic bacteria.

8 1126

9 1127 **Figure 13. 96h of regeneration in *Aplysina cavernicola***

10 1128 A – semi thin section of the completely restored ectosome and upper part of endosome; B –
 11 1129 TEM of the upper part of the restored ectosome; C - the endosome with new choanocyte
 12 1130 chamber; D – the endosome with normal cell composition.

13 1131 ar – archaeocyte, cc – choanocyte chamber, ch – choanocyte, ect – ectosome, end –
 14 1132 endosome, exc- exhalant canal, lo – lophocyte, n – nucleus, sb – symbiotic bacteria, sc –
 15 1133 spherulous cell.

16 1134

17 1135 **Figure 14. Cell proliferation in intact tissues of *Aplysina cavernicola*.** A – ectosome; B –
 18 1136 endosome; C – endosome near the exhalant canal of the aquiferous system; D – DNA-
 19 1137 synthesizing choanocytes with small round to oval nuclei; E – mesohyl DNA-synthesizing
 20 1138 cells with large round nuclei. Cyan – DAPI, green – α -tubulin, magenta – EdU. cc –
 21 1139 choanocyte chamber, end – endosome, ect – ectosome, exc – exhalant canal of the aquiferous
 22 1140 system. White arrowheads marks EdU-positive nuclei of DNA-synthesizing cells. Each image
 23 1141 is a maximum projection, obtained from 55 μ m Z stack.

24 1142

25 1143 **Figure 15. Cell proliferation during *Aplysina cavernicola* body wall regeneration.** A-E –
 26 1144 cell proliferation in the wound area at different stages of regeneration: A – 0-6 hpo; B – 0-24
 27 1145 hpo; C – 24-48 hpo; D – 48-72 hpo; E – 120-144 hpo; F – cell proliferation in intact tissues,
 28 1146 distant from the wound, during regeneration. Cyan – DAPI, magenta – EdU. Dcc –
 29 1147 disintegrating choanocyte chamber, cc – choanocyte chamber. White arrowheads marks EdU-
 30 1148 positive nuclei of DNA-synthesizing cells. Each image is a maximum projection, obtained
 31 1149 from 15 μ m Z stack.

32 1150

33 1151 **Figure 16. Apoptosis during *Aplysina cavernicola* body wall regeneration.** A – 6 hpo; B –
 34 1152 12 hpo; C – 48 hpo. Blue – DAPI, red – TUNEL. White dashed line marks the wound edge,
 35 1153 white arrows – TUNEL-positive nuclei of apoptotic cells. Each image is an optical slice
 36 1154 through the wound area.

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Supporting Figure 1. TEM images of ECM dynamic at the wound surface.

A – intact ; B – 0 hpo ; C - 3 hpo ; D – 6 hpo ; E – 12 hpo ; F – 24 hpo ; G – 48 hpo ; H – 96 hpo.

For Peer Review

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3 **Title**¹ Transdifferentiation and mesenchymal-to-epithelial transition during regeneration
4 in Demospongiae (Porifera)
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36 **Text figures – 16**

37 **Abbreviated title:** Demosponges regeneration
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Abstract

Origin and early evolution of regeneration mechanisms remain among the most pressing questions in animal regeneration biology. Porifera have exceptional regenerative capacities and, as early Metazoan lineage, are a promising model for studying evolutionary aspects of regeneration. Here, we focus on reparative regeneration of the body wall in the Mediterranean demosponge *Aplysina cavernicola*. The epithelialization of the wound surface is completed within two days, and the wound is completely healed within two weeks. The regeneration is accompanied with the formation of a mass of undifferentiated cells (blastema), which consists of archaeocytes, dedifferentiated choanocytes, **anucleated amoebocytes**, and differentiated spherulous cells. The main mechanisms of *A. cavernicola* regeneration are cell dedifferentiation with active migration and subsequent redifferentiation or transdifferentiation of polypotent cells through the mesenchymal-to-epithelial transformation. The main cell sources of the regeneration are archaeocytes and choanocytes. At early stages of the regeneration, the blastema almost devoid of **cell proliferation**, but after 24 hpo and up to 72 hpo numerous **DNA-synthesizing** cells **appear** there. In contrast to intact tissues, where vast majority of **DNA-synthesizing** cells are choanocytes, all EdU-labeled cells in the blastema are **mesohyl cells**. Intact tissues, distant from the wound, retains intact level of cell proliferation during whole regeneration process. For the first time, the apoptosis was studied during the regeneration of sponges. Two waves of apoptosis were detected during *A. cavernicola* regeneration: the first wave at 6-12 hpo and the second wave at 48-72 hpo.

Keywords: demosponges, regeneration, mesenchymal-to-epithelial transformation, blastema, apoptosis, transdifferentiation.

Highlights

- 1) Regeneration in the demosponge *Aplysina cavernicola* is accompanied with the formation of a mass of undifferentiated cells (blastema).
- 2) The main mechanisms of *A. cavernicola* regeneration are cell dedifferentiation with active migration and subsequent redifferentiation or transdifferentiation of polypotent cells - archaeocytes and choanocytes - through the mesenchymal-to-epithelial transformation.
- 3) Apoptosis during regeneration of *A. cavernicola* participate in damaged cells elimination and associated with the extensive ejection of spherulous cells from wound area.

64 Introduction

65 In spite of big interest in various problems concerning origin and early steps of
66 evolution in animal regeneration, morphogenesis, cell turnover etc., up to now there are
67 surprisingly small number of the studies, dealing with ultrastructural, morphogenetic, cell and
68 genetic aspects of sponge reparative regeneration.

69 Phylum Porifera consists of four classes: syncytial Hexactinellida, and cellular
70 Calcarea, Homoscleromorpha and Demospongiae. The last class is the largest and includes
71 about 80% of living sponges. Studies of regeneration in sponges have begun on demosponges
72 (Cavolini, 1785; Vaillant, 1869; Weltner, 1893). However, there are only few papers,
73 concerning ultrastructural description of the morphogenesis and cell behavior in reparative
74 regeneration of sponges. Moreover, three of them dealing with the regeneration of specific
75 “organs” after amputation (oscular diaphragm in *Hippospongia communis* (Thiney, 1972),
76 oscular tube in *Ephydatia fluviatilis* (Sukhodolskaya, 1973), and papillae in *Polymastia*
77 (Boury-Esnault, 1976)). Reparative regeneration of the body wall is described only in **seven**
78 species. Regeneration in *Spongilla lacustris* (Brondsted, 1953), *Halichondria panicea*
79 (Korotkova & Nikitin, 1969), *Geodia barretti* (Hofmann et al., 2003) and *Halisarca caerulea*
80 (Alexander et al., 2015) was studied only with light microscopy. In the case of *H. caerulea*,
81 the light microscopy studies were supplemented with the investigations of the cell
82 proliferation during the regenerative processes (Alexander et al., 2015). **Reparative**
83 **regeneration in *Chondrosia reniformis* was investigated with light and scanning electron**
84 **microscopy (SEM) (Pozzolini et al., 2019). Finally, SEM studies supplemented with time-laps**
85 **recordings were done for regeneration in *Hymeniacidon heliophila* (Coutinho et al., 2017).**

86 At the same time, our complex detailed investigations of reparative regeneration, done
87 with TEM, SEM, **epifluorescent** and light microscopy, immunocytochemistry and time-laps
88 recordings, in homoscleromorphs (Ereskovsky et al., 2015), calcareous sponges (Ereskovsky
89 et al., 2017; Lavrov et al., 2018) and demosponges (Borisenko et al., 2015) show a high
90 diversity of morphogenesis, cell mechanisms, and cell turnover, accompanying these
91 processes.

92 Thus, having comprehensive data about mechanisms of the regeneration for only one
93 species from the huge and very diverse class Demospongiae, we cannot make any
94 generalizations regarding the mechanisms of regeneration of this class of Porifera.

95 Representatives of the genus *Aplysina* are widely distributed in subtropical and tropical
96 coastal waters (Bergquist & Cook, 2002). They are considered proper models in chemical
97 ecology and microbiology (Azevedo et al., 2008; Betancourt-Lozano et al., 1998; Thoms et

1
2
3 98 al., 2004, 2006). *Aplysina cavernicola* is a popular and promising model for various
4
5 99 researches, dealing with the sponge cell composition (Vacelet, 1966, 1967, 1970, 1971, 1975;
6
7 100 Vacelet & Gallissian, 1978), bacterial symbionts (Vacelet, 1975; Fiedrich et al., 1999;
8
9 101 Hentschel et al., 2001; Thoms et al., 2003), three-dimensional skeletal scaffolds (Vacelet,
10
11 102 1971a,b; Garrobe et al. 1973; Ehrlich et al. 2010a, b), biochemistry and secondary metabolites
12
13 103 (D'Ambrosio et al., 1982, 1983; Ciminiello et al., 1997; Reverter et al., 2016), and temporal
14
15 104 variability of secondary metabolism (Reverter et al., 2016). Life cycle researches showed that
16
17 105 *A. cavernicola* is an oviparous sponge whose reproductive period lasts barely one month
18
19 106 (Gallissian & Vacelet, 1976; Reverter et al., 2016).

20
21 107 The present study was aimed at investigation of the reparative regeneration in
22
23 108 Mediterranean demosponge *Aplysina cavernicola* (Vacelet, 1959). The wide range of methods
24
25 109 allow us to make a comprehensive analysis of mechanisms, which contribute to the
26
27 110 regeneration in this species, including cell behavior and migrations, morphogenetic process,
28
29 111 cell proliferation and apoptosis.

30 112

31 113 **Materials and methods**

32 114

33 115 ***Sampling***

34 116 *Aplysina cavernicola* (Vacelet, 1959) (Demospongiae, Verongida) is a perennial
35
36 117 sciaphilous species inhabiting coralligenous formations or the entrance of submarine caves
37
38 118 generally between 8 to 60 m in the Mediterranean Sea (Figure 1). It presents a typical
39
40 119 yellowish color. For regeneration experiments *A. cavernicola* specimens were collected by
41
42 120 SCUBA diving in September-November 2017, July-August and October 2018 and March
43
44 121 2019 near Maire Island, Marseille (43.2096° N; 5.3353° E) at a depth of 12 - 15 m. Collected
45
46 122 sponges were maintained in a 100 l laboratory aquarium with running natural seawater at a
47
48 123 temperature of 15-16°C for 36 hours for sponge adaptation.

49 124

50 125 ***Surgical operations***

51 126 Two type of surgical operations have been conducted: i) *in situ*, and ii) in laboratory.
52
53 127 For *in situ* operations six individuals were used. The experiment was performed in June 2018
54
55 128 at the site of sponge sampling. In each sponge wounds were made in a wall of a cylindrical
56
57 129 outgrowth using a sharp stainless dissecting scalpel. The wounds had a uniform size of 3 cm²
58
59 130 in area and 1 cm deep. Each wound was measured and photographed at $t = 0, 2, 7, 12,$ and 32
60
61 131 days post operation, using digital camera Nikon D300 equipped with waterproof camera

1
2
3 132 housing SUBAL ND300 and flash INON Z-240.

4
5 133 Surgical operations in laboratory were performed as an excision of a small part
6
7 134 (approximately 0.3-0.5x0.3-0.5 cm) of the body wall at the base of a cylindrical outgrowth
8
9 135 (Figure 2). A total of 24 individuals were used in the body wall regeneration experiments
10
11 136 (Supporting Table 1).

12 137 The surgical operations were done manually under a stereomicroscope using scalpel.
13
14 138 After the operations the sponges with excised body wall were maintained in a 100 l laboratory
15
16 139 aquarium with running natural seawater at a temperature of 15-16°C. The sponges were
17
18 140 inspected and photographed using a stereomicroscope Leica M165FC (Leica) equipped with a
19
20 141 digital camera Leica DFC 320 (Leica) and LAS Store and Recall v.4.1 software (Leica). The
21
22 142 observations were done at 3, 6, 12, 18, 24, 36, 48, 72, 96, and 120 hours post operation (hpo).

23
24 143

24 144 ***Light and electron microscopy***

25 145 Specimens were fixed overnight at 4°C by 2.5% glutaraldehyde (Ted Pella) on 0.2M
26
27 146 cacodylate buffer (pH 7.4) and post-fixed for 2 h with 1% OsO₄ (Spi Supplies) on the same
28
29 147 buffer at room temperature (RT). Between fixation and post-fixation specimens were twice
30
31 148 rinsed with cacodylate buffer for 30 min. Finally, specimens were dehydrated in an ethanol
32
33 149 series at RT and stored in 70% ethanol at 4°C.

34 150 For semi-thin sections and transmission electron microscopy (TEM) specimens were
35
36 151 embedded in Araldite (Sigma-Aldrich) epoxy embedding media according to the
37
38 152 manufacturer instructions. Semi-thin sections (1 µm) were cut on a Reichert Jung
39
40 153 ultramicrotome (Reichert) and Ultramicrotome PowerTome XL (RMC Boeckeler) and then
41
42 154 stained with 1% toluidine blue – 0.2% methylene blue mixture. The semi-thin sections were
43
44 155 studied under a WILD M20 microscope (Wild). Digital photos were taken with a Leica
45
46 156 DMLB microscope (Leica) using Evolution LC color photo capture system
47
48 157 (MediaCybernetics).

49 158 Ultrathin sections (60–80 nm) were cut with a Leica UCT6 and an Ultramicrotome
50
51 159 PowerTome XL, equipped with a Drukkert 45° diamond knife, and contrasted with 4%
52
53 160 aqueous uranyl acetate. Ultrathin sections were studied under Zeiss-1000 (Carl Zeiss)
54
55 161 transmission electron microscope.

56 162 For scanning electron microscopy (SEM), fixed specimens were critical-point-dried,
57
58 163 sputter-coated with gold-palladium, and observed under Hitachi S 570 (Hitachi) microscope.

59 164

60 165 ***Spherulous cell counting***

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3 166 Spherulous cells were counted on images of semi-thin sections of intact sponge tissues
4 and regenerating specimens at 6, 12, 24, 48, and 96 hpo. The images were obtained with a
5 167 Leica DMLB microscope (Leica) at 40x magnification using Evolution LC color photo
6 168 capture system (MediaCybernetics). At each stage three images, arising from three
7 independent individuals, were used for counting. Spherulous cells were counted in the
8 169 approximate 50- μ m thick lane beneath the exopinacoderm in intact sponges or beneath wound
9 surface in regenerating individuals. The area of the studied lanes was measured for each
10 170 image, and number of spherulous cells were extrapolated for an area of 1 mm². Cell counting
11 and area measuring were done with ImageJ v.1.48 software (National Institute of Health). For
12 171 each stage mean value and standard deviation were calculated (Supporting Table 4).
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20 176 Statistical analysis was performed in R (R Core Team, 2019) with basic package “stats”
21 ver. 3.6.0 (R Core Team, 2019) and additional packages “agricolae” ver. 4.2-0 (de
22 177 Mendiburu, 2019), “car” 3.0-3 (Fox & Weisberg, 2019) and graphic package “ggplot2” ver.
23 178 3.3.1 (Wickham, 2019). To analyze the results, analysis of variance (ANOVA) was performed
24 to evaluate differences for spherulous cell count in intact tissues and at different stages of
25 179 regeneration. For ANOVA we prerequisitely performed box-cox transformation with $\lambda=0$ to
26 normalize our data, and Leven’s test for homogeneity of variances ($p = 0.3744$). For pairwise
27 180 comparisons, we performed Tukey’s honestly significant test and Duncan’s multiple range
28 test from “agricolae” package with 0.95 confidence level (Supporting Table 4).
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38 186 ***Cell proliferation investigations***

39 187 A total of 27 individuals with the excised body wall were used in cell proliferation
40 studies (Supporting Table 2). The 5-Ethynyl-2'-deoxyuridine (EdU) (Thermo Fisher
41 188 Scientific), which incorporates in nuclear DNA during its synthesis in S-phase and marks
42 DNA-synthesizing cells, was used as a label for cell proliferation. The EdU stock solution
43 189 was prepared in DMSO (MP Biomedicals). The optimal EdU concentration and incubation
44 time were elucidated during the preliminary studies with the intact tissues of *Aplysina*
45 190 *cavernicola*.
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51 194 Labeling of DNA-synthesizing cells in the regenerating sponges were conducted during
52 the following time periods: 0-6, 6-12, 0-24, 24-48, 48-72, 120-144 hpo. The EdU
53 195 concentration in the experiments with 6-hour incubation period was 600 μ M and in the
54 experiments with 24-hour incubation period – 200 μ M. Three individuals were used at each
55 196 time period. Three additional sponges were cultured in FSW without the EdU and served as
56 negative technical controls (Supporting Table 2).
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2
3 200 The cell proliferation was also studied in the intact tissues of *A. cavernicola*. Three
4 201 individuals were incubated 6 hours in FSW with 600 μ M EdU and three individual – 24 hours
5 202 in FSW with 200 μ M EdU. One individual was cultured in FSW without the EdU and served
6 203 as a negative technical control (Supporting Table 2). The mode of EdU incubation did not
7 204 significantly influence the pattern of the staining of DNA-synthesizing cells: after both types
8 205 of incubation sponge tissues show essentially the same amount and localization of the DNA-
9 206 synthesizing cells.

15 207 During the EdU incubation, intact and regenerating sponges were cultivated in glass
16 208 vessels with 200 ml FSW supplemented with required amount EdU at 13°C.

18 209 After the incubation period, all individuals were rinsed twice with FSW and fixed with
20 210 4% PFA (Sigma-Aldrich) in PBS (Amresco, Inc.) for 12-15 hours at 4°C. Fixed specimens
21 211 were rinsed with PBS and the Click-iT reaction were performed in the following mixture: 4
22 212 mM CuSO₄ (ChimMed), 20 mg/ml Sodium L-ascorbate (Sigma-Aldrich) and 10 μ M Sulfo-
23 213 Cyanine3 Azide (Lumiprobe) in PBS. Finally, the specimens were rinsed several times with
24 214 PBS and stained with DAPI (Sigma-Aldrich).

29 215 Stained specimens were mounted in 90% glycerol-DABCO (Sigma-Aldrich) and
30 216 studied with a CLSM Nikon A1 (Nikon) using lasers with 405 nm, 488 nm and 546 nm
31 217 wavelength. The tissues beneath the wound surface and tissues no less than 1 cm away from
32 218 the wound were studied in each regenerating specimen.

36 219 The obtained Z-stacks and images were processed with ImageJ v.1.48 software
37 220 (National Institute of Health). Nuclei measuring were done on separate optical slices with NIS
38 221 Elements Viewer v. 4.5 (Nikon) and JR Screen Ruler v. 1.5 (Spadix Software). For all
39 222 measurements mean value and standard deviation were calculated.

223 224 *Apoptosis investigation*

225 Four individuals were used for studies of apoptosis during regeneration and in intact
226 227 sponge tissues (Supporting Table 3). The studies were performed using the In Situ Cell Death
228 229 Detection Kit (Roche) or Click-iT Plus TUNEL Kit (Thermo Fischer Scientific). Both kits
230 231 detect apoptotic cells using the TUNEL assay, i.e. by attaching labeled nucleotides to double-
232 233 stranded DNA breaks that occur at the later stages of apoptosis.

230 Intact tissue and wounded areas at 6, 12, 24 and 48 hpo were fixed at 4°C by 4% PFA
231 (Sigma-Aldrich) on PBS (Amresco, Inc.). Fixed specimens were rinsed with PBS and treated
232 according to the manufacturer instructions for apoptotic cell visualization. Finally, the
233 specimens were rinsed several times with PBS and stained with DAPI (Sigma-Aldrich).

234 Samples, incubated with DNase I recombinant purified from bovine pancreas (Thermo
235 Fisher Scientific) prior to the TUNEL reaction, were used as positive technical controls.
236 Samples, incubated without the TdT enzyme during the TUNEL reaction, were used as
237 negative technical controls.

238 Stained specimens were mounted in Mowiol (12%) or 90% glycerol-DABCO (Sigma-
239 Aldrich) and studied with a confocal microscope TCS-SPE (Leica) or CLSM Nikon A1
240 (Nikon) using lasers with 405 nm, 488 nm and 546 nm wavelength. The tissues beneath the
241 wound surface and tissues no less than 1 cm away from the wound were studied in each
242 regenerating specimen.

243

244 ***Field Study Permissions***

245 No specific permissions were required for these locations because the study was done
246 outside national parks, private lands or protected areas. We declare that the field studies did
247 not involve endangered or protected species.

248

249 **Results**

250

251 **Intact sponge morphology and histology**

252 The body of *Aplysina cavernicola* has a branchy shape, with each cylindrical branch
253 having 1-2 cm in diameter (Figure 1). Sponge tissues are dense and elastic. The sponge has
254 leuconoid organization of aquiferous system (numerous small choanocyte chambers, scattered
255 in the mesohyl). The skeleton represented exclusively by organic (spongin) fibers, covered
256 with chitin (Ehrlich et al., 2010a), thus the surgical operations are easily conducted.

257 The body is composed of the peripheral ectosome and the internal endosome, bearing
258 numerous choanocyte chambers (Figure 3A). The ectosomal region is up to 30 μm thick and
259 consists of three layers: (1) external parts of the T-shaped exopinacocytes, connected by non-
260 specialized cell junctions (Figure 3B,C) and covered by an acellular cuticle; (2) layer
261 containing collagen fibrils, cell bodies of exopinacocytes and scattered spherulous cells; and
262 (3) the inner layer, consisting of condensed collagen fibrils and spherulous cells. The
263 endosome (Figure 3A) composes the major part of the sponge body. It includes choanocyte
264 chambers, consisted of flagellated choanocytes, aquiferous canals, lined by endopinacocytes,
265 (Figure 3D,E) and the mesohyl with the skeleton, abundant symbiotic bacteria and scattered
266 specialized sponge cells.

267 Populations of free cells in the mesohyl of *A. cavernicola* include: lophocytes,
 268 archaeocytes, pocket cells, contractile cells (myocytes) (Figure 3F,H), spherulous cells at
 269 different stages of their maturation with two principal morphotypes: larger cells with clear
 270 inclusions (Figures 3C, 4G) and smaller ones with dense inclusions (Figure 4D,H),
 271 bacteriocytes, microgranular cells, spongocytes (Figure 4A-F) (Vacelet, 1966, 1967, 1970,
 272 1971, 1975; Vacelet & Gallissian, 1978).

273

274 **Regeneration**

275 In spite of **minor** individual differences wound healing in *Aplysina cavernicola*, which
 276 is expressed in the epithelialization of the wound surface, is completed within two – six days
 277 (Figure 1). **During our observations we did not reveal any significant differences in the onset**
 278 **of the stages and course of the regeneration, as well as in the morphogenesis accompanying it,**
 279 **across studied individuals.** The regeneration ends within two weeks, when the wound is
 280 completely healed: only a small depression on the surface remains in its place.

281 At the histological level the observed regeneration processes can be subdivided into
 282 three stages: 1) internal milieu isolation – formation of **a clot** (3 – 12 hpo), 2) wound healing –
 283 epithelization (12 – 24 hpo), and 3) restoration of ectosome and endosome (36 – 96 hpo).

284 For detailed description of morphogenesis and cell behavior, accompanying the
 285 regeneration, we propose to subdivide a wound and tissue around the wound on several areas
 286 (Figure 5):

- 287• Wound – a break in the continuity of any bodily tissue due to injury.
- 288• Wound area – tissues directly adjacent to an excised part of the sponge body; their structure is
 289 severely disrupted during the surgery.
- 290• Edge of the wound – peripheral parts of the wound, which is in direct contact with intact
 291 tissues.
- 292• Regeneration area – an area of the sponge body (tissue), which is not directly affected by
 293 surgery, but in which anatomical structures (choanocyte chambers, canals of aquiferous
 294 system and skeleton) are reorganized, and the normal composition and distribution of cells
 295 disrupted due to their participation (dedifferentiation, transdifferentiation and migration) in
 296 the regenerative processes. The dimension of this area could vary, depending on size and type
 297 of the injury and on individual characteristics of a sponge.
- 298• Intact issues – ectosome and endosome areas that are not affected by surgery and are not
 299 directly involved in regeneration and retaining the normal organization.

1
2
3 300 ***Stage I – Internal milieu isolation***

4
5 301 Immediately after the surgical excision of ectosome with the directly adjacent endosome,
6
7 302 the wound surface retracts, leaving the surface of the intact ectosome protruding around the
8
9 303 edges of the wound. The ectosome and the upper areas of endosome are destroyed in the
10
11 304 wound area.

12 305 During the first 3 hours post operation (hpo), the wound surface is covered with
13
14 306 exudate, cell debris and numerous symbiotic bacteria. The extracellular matrix (ECM) does
15
16 307 not show any signs of a condensation (Supporting Figure 1A-C). All cells in the wound area
17
18 308 undergo morphological modifications. The epithelial cells - endopinacocytes and choanocytes
19
20 309 of choanocyte chambers, begin losing contacts with adjacent cells in their epithelial layers and
21
22 310 change their shape from trapeziform (choanocytes) and flat (endopinacocytes) to spherical or
23
24 311 amoeboid (Figure 6). These dedifferentiated cells mix with the mesohyl cell population.
25
26 312 During transformation of the choanocytes, their collar of microvilli and flagellum are
27
28 313 resorbed (Figure 6D). However, the elements of the flagellar apparatus (the basal body and
29
30 314 accessory centriole located near the nucleus) persist in the transformed cells for
31
32 315 approximately two days and serve as the natural marker of dedifferentiated choanocytes. The
33
34 316 dedifferentiated endopinacocytes have no specific morphological characteristics and therefore
35
36 317 these cells cannot be distinguished from other mesohyl cells. The non-secreting cells of the
37
38 318 mesohyl (archaeocytes, lophocytes, dedifferentiated choanocytes and endopinacocytes)
39
40 319 actively phagocytosed cell debris, symbiotic and invasive microbes, including diatoms in the
41
42 320 wound area (Figure 6). Thus, all cells in the wound area, except the spherulous cells, are filled
43
44 321 with large phagosomes. The ectosome, surrounding the wound slightly contracts and bends
45
46 322 inward. After 3 hpo an active migration of the mesohyl cells and dedifferentiated choanocytes
47
48 323 towards the wound surface begins from wound area, as these cells assume an elongated shape
49
50 324 with the long axis, perpendicular to the wound surface (Figure 6). There are no changes in the
51
52 325 regeneration area in this period.

53
54 326 At 6 hpo, the wound surface is aligned and becomes flat and smooth. It is covered by a
55
56 327 thick layer of ECM, containing fragments of cells, dispersed symbiotic bacteria and few
57
58 328 spherulous cells (some of which are beginning their dedifferentiation) (Figure 7A; Supporting
59
60 329 Figure 1D). The amount of the spherulous cells in the wound area at this stage of the
330
331 regeneration significantly decreases and is approximately 2,8-fold lower than in the ectosome
332
333 of intact sponges (Figure 8; Supporting Table 4). This structure can be referred as a
regenerative clot, by analogy with other animals (Carlson, 2007). The thickness of the clot
is from 12 to 20 μm .

1
2
3 334 The cells of wound area have numerous small or few large phagosomes, which
4 335 contained fragments of the spherulous and granular cells (Figure 7B-F). Such cells could be
5 336 found not only in the upper part of the wound, but also in the deeper zone up to 100 μ m
6 337 beneath the wound surface. There still no visible changes in the regeneration area at this
7 338 stage.

8 339 At 12 hpo a regenerative clot at the wound surface is getting thinner, and wound surface
9 340 become aligned, flat and smooth (Figure 9A). The peripheral parts of the wound are clearly
10 341 limited by the flat outgrowths of intact exopinacocytes (Figure 9F).

11 342 In the wound area, cell distribution begins ordering, and ECM condensation occurs
12 343 (Supporting Figure 1E). The number of dedifferentiated choanocytes and endopinacocytes,
13 344 archaeocytes, and lophocytes increases in this area in comparison with the previous period of
14 345 regeneration (Figure 9B-E). Majority of these cells migrated from regenerated area. In
15 346 contrast, the number of the spherulous cells shows insignificant variations and remains
16 347 approximately the same as at 6 hpo (Figure 8; Supporting Table 4). Some of these spherulous
17 348 cells undergo the dedifferentiation, which is accompanied by the release of the spherules from
18 349 the cells. The amount of free mesohyl and concentration of the symbiotic bacteria decrease in
19 350 comparison with previous periods of regeneration (Figure 9C, D).

20 351 Simultaneously, the migration of the amoeboid cells (archaeocytes, dedifferentiated
21 352 choanocytes) from the regeneration area to the wound area and wound surface proceeds
22 353 (Figure 9C,E), and some of the migrating cells reach the wound surface, where they become
23 354 oriented with the long axis parallel to the wound surface (Figure 9B). Also first elongated
24 355 contractile cells (myocytes) appear in the regeneration area.

25 356

26 357 ***Stage II. Wound healing - epithelization (12 – 24 hpo)***

27 358 At 24 hpo a cell mass, consisting of heterogenous dedifferentiated and undifferentiated
28 359 (archaeocytes) cells, is formed under the upper part of the wound area. We considered it as a
29 360 blastema, as structurally it resembles blastemas formed during regeneration of other animals.
30 361 Blastema occupies the wound area and the upper part of the regeneration area.

31 362 Simultaneously, the upper part of the wound area becomes similar to intact sponge
32 363 ectosome, showing structured ECM with collagen fibrils (Figure 10A,B; Supporting Figure
33 364 1F). The wound surface is mosaically covered with very thin superficial outgrowths of
34 365 developing T-shaped exopinacocytes, arising from blastema cells of the heterogeneous origin
35 366 (Figure 10B,C,E). New exopinacocytes do not yet form close contacts with each other. In
36 367 some places submerged nucleated bodies of these cells are found. New exopinacocytes, in

1
2
3 368 contrast to the cells located deeper under the wound surface, have only few phagosomes.
4
5 369 There is no directed movement (contraction or creeping) of the intact exopinacoderm
6
7 370 surrounding the wound.

8 371 The number of spherulous cells in the wound area significantly increases in
9
10 372 approximately 2,3-folds in comparison with the previous stage of regeneration (Figure 8;
11
12 373 Supporting Table 4). These cells show typical appearance (Figure 10B). Their shape is oval,
13
14 374 not amoeboid, as in the earlier stages of regeneration. In contrast, near the wound surface, the
15
16 375 number of dedifferentiated choanocytes, archaeocytes and spherulous cells decreases (Figure
17
18 376 10C-F). The lophocytes, granular cells, and myocytes are absent from this zone. The
19
20 377 choanocyte chambers or their fragments completely disappear from the regeneration area at
21
22 378 this stage of regeneration. They occur at a depth of about 200 μm , as in intact sponge.

22 379

23 380 *Stage III. Restoration of ectosome and endosome (36 – 96 h)*

24 381 At 48 hpo the ectosome of the regenerate is completely restored, but superficial cuticle,
25
26 382 characteristic for intact sponge, is still absent (Figure 11A,B; Supporting Figure 1G). The
27
28 383 wound epithelization is finished by new exopinacocytes, arose from the heterogeneous
29
30 384 dedifferentiated cells population and archaeocytes. Some of new exopinacocytes clearly show
31
32 385 their origin from the dedifferentiated choanocytes, as they still bear flagellar basal apparatus
33
34 386 (Figure 11C). According to the orientation of this flagellated complex, it can be assumed that
35
36 387 the basal part of the former choanocyte is flattened. Archaeocytes, reaching the wound, flatten
37
38 388 and assume position parallel to the surface (Figures 10E,F, 11D). New exopinacocytes have
39
40 389 normal T-shape and cell contacts.

41 390 During formation of the exopinacoderm active elimination of small apoptotic
42
43 391 spherulous cells with compact inclusions and cells, filled with big heterophagosomes, begins
44
45 392 (Figure 11E, F). However, the number of the spherulous cells shows insignificant variations
46
47 393 and remains approximately the same as at 24 hpo (Figure 8; Supporting Table 4).

48 394 During this stage, the choanocyte chambers and aquiferous canals of the endosome are
49
50 395 restored by association of previously disaggregated choanocytes and endopinacocytes,
51
52 396 respectively (Figure 12). Archaeocytes also participate in the development of new choanocyte
53
54 397 chambers (Figure 12D). Cells that form new structure of aquiferous system contact each other
55
56 398 and connect by interdigitations. Individual choanocytes form groups of cells, forming
57
58 399 structures less compact than in the intact choanocyte chamber (Figure 12D-F). These cells
59
60 400 gradually transform from mesenchymal morphology to epithelial.

1
2
3 401 At 96 hpo, the regeneration is almost complete: the ectosome obtains the cuticle, while
4 402 the endosome of the regeneration area begins to recover: choanocyte chambers and aquiferous
5 403 canals are gradually developing (Figure 13; Supporting Figure 1H). The number of
6 404 spherulous cells in the regenerated ectosome significantly increases, reaching the intact level
7 405 (Figure 8; Supporting Table 4). The free cells of the mesohyl, with the exception of
8 406 specialized secretory cells, still contain phagosomes.

407 408 ***Cell proliferation***

409 The DNA-synthesizing (EdU-labelled) cells are irregularly distributed in the intact
410 tissues of *A. cavernicola* (Figure 14). Numerous DNA-synthesizing cells are located in the
411 sponge endosome (Figure 14B). The majority of these cells are choanocytes with small
412 ($3.87 \pm 0.46 \mu\text{m}$; $n=45$) round to oval nuclei (Figure 14D). Nevertheless, some mesohyl cells
413 with large ($4.84 \pm 0.44 \mu\text{m}$; $n=50$) round nuclei are also labelled by EdU (Figure 14E). These
414 mesohyl DNA-synthesizing cells occurs all over sponge mesohyl: in the ectosome, endosome
415 and tissues, adjacent to the large exhalant canals of the aquiferous system, which are
416 structurally similar to the ectosome. The ectosome and tissues, adjacent to the large exhalant
417 canals contain only few such mesohyl DNA-synthesizing cells (Figure 14A, C).

418 During the regeneration, the pattern of the cell proliferation dramatically changes
419 (Figure 15). Immediately after the surgical operation, the level of the cell proliferation in the
420 tissues, adjacent to the wound, decreases sharply: at 0-6 hpo, DNA-synthesizing cells are
421 completely absent in a 100- μm zone below the wound surface (Figure 15A); at 6-12 hpo and
422 0-24 hpo, rare DNA-synthesizing cells appear in the tissues, located 30-40 μm below the
423 wound surface. The majority of the labelled cells are choanocytes from disintegrating
424 choanocyte chambers, however some mesohyl cells are also labelled. Similarly, to the intact
425 tissues, the labelled choanocytes have small ($3.85 \pm 0.4 \mu\text{m}$; $n=13$) round to oval nuclei, while
426 labelled mesohyl cells are characterized by large ($4.79 \pm 0.37 \mu\text{m}$; $n=16$) round nuclei.

427 After 24 hpo, further changes in the cell proliferation pattern occurs in the regeneration
428 area. The samples at 24-48 hpo and 48-72 hpo show a similar pattern (Figure 15B, D): in a
429 100- μm zone below the wound surface, numerous DNA-synthesizing cells occur. Some of
430 these cells are located just beneath the wound surface. Virtually all labeled cells are located in
431 the mesohyl, since the choanocyte chambers disappear in the tissues, adjacent to the wound,
432 by this time. These DNA-synthesizing cells have large ($4.99 \pm 0.39 \mu\text{m}$; $n=16$) round nuclei,
433 similar to EdU-labelled mesohyl cells from intact tissues. Occasionally, single DNA-

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3 434 synthesizing choanocytes occur in the tissues, located 60-70 μm below the wound surface,
4
5 435 where intact choanocyte chambers could be retained.

6 436 At 120-144 hpo, after the recovery of ectosome and endosome structure in the
7
8 437 regeneration area, the cell proliferation returns to the normal state, showing the intact pattern
9
10 438 with majority of DNA-synthesizing cells, occurring in choanocyte chambers. However, the
11
12 439 level of cell proliferation is still lower than in intact tissues (Figure 15E).

13 440 In the tissues, distant from the wound, the intact cell proliferation pattern persists during
14
15 441 the whole regeneration process (Figure 15F).

16
17 442

18 443 *Apoptosis*

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20 444 We found no apoptotic cell in the intact tissue of *A. cavernicola*.

21
22 445 Two waves of apoptosis occur during the regeneration process. The level of apoptosis is
23
24 446 low during both waves with few apoptotic cells, located only at the upper part of the wound.
25
26 447 In the endosome under the wound and in tissues distant from the zone of regeneration
27
28 448 apoptotic cells are completely absent.

29 449 The first wave is associated with the early stages of regeneration. Apoptotic cells appear
30
31 450 at 6 hpo, and their number gradually decline during further regeneration (Figure 16A). At 12
32
33 451 hpo only single apoptotic cells occur at the upper parts of the wound area (Figure 16B), while
34
35 452 at 24 hpo they are virtually absent. Apparently, this wave of apoptosis participates in the
36
37 453 elimination of damaged cells from the wound area.

38 454 The second wave of apoptosis occurs at 48 hpo, when relatively numerous apoptotic
39
40 455 cells appears at the upper parts of the wound area (Figure 16C). This wave is probably
41
42 456 associated with the active elimination of cells through the emerging exopinacoderm during
43
44 457 later stage of regeneration (Figure 11E,F).

45 458

46 459 **Discussion**

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48 460

49 461 **1 General**

50 462 *In vivo* observations

51
52 463 Sponges are well known for their capacity to regenerate not only small body parts, but
53
54 464 also after substantial partial mortality and damage. *In situ* monitoring of naturally and
55
56 465 experimentally generated wounds confirms high recovery capacity of sponges from different
57
58 466 taxa and of different growth forms (Connes, 1966, 1968; Ayling, 1983; Hoppe, 1988;
59
60 467 Duckworth, 2003; Henry & Hart, 2005; Wulff, 2010, 2013). These studies also discovered

1
2
3 468 that regeneration can be influenced both by characteristics of the wound and by inherent
4
5 469 characteristics of particular species of sponges.

6 470 Our observations *in situ* showed that wound epithelialization under natural conditions
7
8 471 occurs in *Aplysina cavernicola* during two days. Other investigations provided with different
9
10 472 demosponges showed the same results (Maas, 1910; Brondsted, 1953; Connes, 1966, 1968;
11
12 473 Korotkova & Nikitin, 1969; Korotkova et al., 1983; Diaz, 1979; Hoppe, 1988; Hofmann et al.,
13
14 474 2003; Wulff, 2010, 2013; Alexander et al., 2014; Borisenko et al., 2015; Pozzolini et al.,
15
16 475 2019).

17 476 Previous investigations of regeneration demonstrated that in many individuals of the
18
19 477 same species the amount of damage, type of damage, size of the sponge, and location on the
20
21 478 individual sponge can influence recovery, and even susceptibility to further damage by other
22
23 479 agents (Henry & Hart, 2005; Wulff, 2010, 2013). However, we find small individual
24
25 480 variability during regeneration of *A. cavernicola*, probably because the wounds were applied
26
27 481 in the same areas of the sponges and had the same size.

28 482

29 483 **Histological observations**

30 484 In this work we showed that regeneration processes in *Aplysina cavernicola* in general
31
32 485 follow the stages similar to those described in other massive sponges with leuconoid
33
34 486 aquiferous system - Demospongiae and Homoscleromorpha (Brondsted, 1953; Korotkova &
35
36 487 Nikitin, 1969; Diaz, 1979; Hofmann et al., 2003; Borisenko et al., 2015; Ereskovsky et al.,
37
38 488 2015; Pozzolini et al., 2019), and in various eumetazoans (Korotkova, 1997; Carlson, 2003).
39
40 489 Therefore, *A. cavernicola* regeneration includes three main stages: 1) internal milieu isolation
41
42 490 - formation of “regenerative clot”, 2) wound healing - epithelization, and 3) restoration of
43
44 491 damaged structures - ectosome and endosome. The wound epithelization in *A. cavernicola*
45
46 492 occurs without formation of the regenerative membrane, a structure, characteristic for
47
48 493 regeneration of asconoid and syconoid calcareous sponges (Jones, 1957; Korotkova, 1961,
49
50 494 1962; Ereskovsky et al., 2017; Lavrov et al., 2018).

51 495 The main mechanisms of *Aplysina cavernicola* reparative regeneration of the body wall
52
53 496 are (1) cell dedifferentiation with their subsequent redifferentiation, (2) transdifferentiation,
54
55 497 and (3) active migration of polypotent cells (archaeocytes and choanocytes) to the wound.
56
57 498 The same basic mechanisms are also characteristic for regeneration in other studied
58
59 499 demosponges: *Halichondria panicea* (Korotkova & Nikitin, 1969), *Suberites massa* (Diaz,
60
500 1979), *Halisarca dujardinii* (Borisenko et al., 2015), and *Suberites domuncula* (our
501 unpublished results). However, these mechanisms strongly differ from those, participating in

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2
3 502 regeneration process in calcareous sponges and homoscleromorphs, which show clear
4 503 epithelial organization with specialized cell junctions in the epithelia (Ereskovsky et al., 2015;
5 504 Lavrov et al., 2018).

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8 505

10 506 **2. Morphogenetic mechanisms of *A. cavernicola* regeneration**

11 507 Main morphogenesis during body wall regeneration in *A. cavernicola* is mesenchymal-
12 508 to-epithelial transformation (MET). Indeed, at 12 hpo archaeocytes and dedifferentiated
13 509 choanocytes as well as the spherulous cells begin to move toward wound area, where they
14 510 form a blastema-like structure. Part of external blastema cells begins to flat and form cover
15 511 sponge epithelium (exopinacoderm) through the MET.

16 512 However, experimentally induced (by surgical operation) epithelial-to-mesenchymal
17 513 transition (EMT) proceeds MET in *A. cavernicola*. During this process choanocytes and
18 514 endopinacocytes leave the choanocyte chambers and the canals of aquiferous system,
19 515 correspondingly, and move into the mesohyl, dedifferentiating and assuming an amoeboid
20 516 shape. This morphogenesis is one of central for the creation of numerous organs and complex
21 517 tissues during embryonic development, asexual reproduction, and regeneration and has been
22 518 well described in Eumetazoa (Keller et al., 2003; Lim & Thiery, 2012). EMT is also well
23 519 known during the normal ontogeny of sponges and occurs in the course of embryonic
24 520 development, and larval metamorphosis (reviewed in Ereskovsky et al. 2013). This
25 521 morphogenesis was described during the first stages of regeneration of other investigated
26 522 demosponges and homoscleromorphs (Korotkova & Nikitin, 1969; Borisenko et al., 2015;
27 523 Ereskovsky et al., 2015).

28 524 After wound epithelization, the restoration of elements of the aquiferous system begins.
29 525 Both choanocyte chambers and aquiferous system canals are formed through MET: separate
30 526 amoeboid cells coalesce into groups and gradually transform from mesenchymal
31 527 morphology to epithelial-like cells (choanocytes and endopinacocytes, respectively).

32 528 The same MET mechanism is characteristic for exopinacoderm and choanoderm
33 529 formation during regeneration of other investigated demosponges: *Halichondria panicea*
34 530 (Korotkova & Nikitin, 1969), *Halisarca dujardini* (Borisenko et al., 2015), and
35 531 basopinacoderm formation in *Hymeniacidon heliophila* regeneration (Coutinho et al., 2017).

36 532 It is fundamentally different from the regeneration in Calcarea and Homoscleromorpha,
37 533 in which epithelial morphogenesis, e.g. flattening and spreading of epithelial layers, plays a
38 534 leading role in the restoration of main structures (exopinacoderm and elements of aquiferous
39 535 system) (Ereskovsky et al., 2015; Lavrov et al., 2018).

536

3. Blastema

537
538 In regenerative biology blastema is a mass of undifferentiated cells, located beneath the
539 wound and produced by the dedifferentiation of many cell types and/or migration of
540 polypotent cells. In some cases, differentiation of blastema cells, and further reconstruction
541 the lost part of the body could be accompanied with cell proliferation. In this case, the mitotic
542 rate of the blastema slows down as the structure grows, and it ceases completely when the
543 new structure reaches the original size (Santos-Ruiz et al., 2002; Carlson, 2007; Tsonis, 2008;
544 Vervoort, 2011).

545 The formation of a concentration of cells under the wound in the middle stages of a
546 body wall regeneration has been shown for several demosponges (Thiney, 1972; Boury-
547 Esnault, 1976; Borisenko et al., 2015; Coutinho et al., 2017). This cellular concentration
548 consists of archaeocytes and choanocytes, which are principal stem cells of demosponges
549 (Funayama, 2018), dedifferentiated cells (e.g., pinacocytes) and specialized differentiated
550 cells (e.g., gray cells) (Thiney, 1972; Boury-Esnault, 1976; Borisenko et al., 2015; Coutinho
551 et al., 2017). This structure could be referred as a blastema, characteristic for a regeneration in
552 many animals.

553 Similarly, in the current study, we refer to the undifferentiated cell mass beneath a
554 wound during the end of second and beginning of third stages of regeneration in *A.*
555 *cavernicola* as a blastema. In *A. cavernicola*, it consists of archaeocytes, dedifferentiated
556 choanocytes, mixed population of anucleated amoebocytes (dedifferentiated pinacocytes,
557 myocytes, lophocytes), and differentiated spherulous cells.

558 At early stages of regeneration, the blastema almost devoid of DNA-synthesizing cell,
559 but after 24 hpo and up to 72 hpo numerous DNA-synthesizing cells appears there. However,
560 even during this period the level of cell proliferation in the blastema is much lower in
561 comparison with intact sponge tissues. In contrast, intact tissues distant from the wound
562 retains intact level of cell proliferation during whole regeneration process. The similar pattern
563 of cell proliferation has been described during body wall regeneration of *Halisarca caerulea*
564 (Alexander et al., 2014) and *Halisarca dujardini* (Borisenko et al., 2015), for which
565 decreased proliferation in the wound area is characteristic.

566 Moreover, in contrast to intact tissues of *A. cavernicola*, where vast majority of DNA-
567 synthesizing cells are choanocytes, all EdU-labeled cells in the blastema are mesohyl cells, as
568 blastema lacks choanocyte chambers. These EdU-labeled cells could arise directly in blastema
569 through cell divisions or be EdU-labeled migrating descendants of proliferating cells in intact

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3 570 tissues, distant from the wound. This issue requires further investigations, using additional
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5 571 markers, like antibodies against phospho-histone H3 for revealing cells in M-phase and cell
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7 572 tracers for the visualization of cell migrations.

8
9 573 Importantly, studied sponges from classes Calcarea (*Leucosolenia cf. variabilis*)
10 574 ([Lavrov et al., 2018](#)) and Homoscleromorpha (*Oscarella lobularis*) ([Ereskovsky et al., 2015](#))
11 575 demonstrate a distinct mode of regeneration without blastema formation. In these sponges
12 576 regeneration occurs due to the local remodeling of intact tissues, adjacent to the wound.
13
14 577 Moreover, the cell proliferation is neither affected nor contributes to the regeneration at any
15
16 578 stage.

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18 579
19
20 580 **4. Dedifferentiation, transdifferentiation and cell sources in *A. cavernicola***
21
22 581 **regeneration**

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24 582 Transdifferentiation is the transformation of one type of already differentiated cell into
25
26 583 another type of differentiated cell. In some cases, transdifferentiation is accompanied by cell
27
28 584 division, while in other cases it is not (Shen et al., 2004).

29 585 Sponge cell transdifferentiation is likely a driving force [accompanying](#) their restoration
30 586 processes (Korotkova, 1997; Lavrov & Kosevich, 2014; Adamska, 2018). However,
31 587 transdifferentiating cell types and mechanisms of this process vary in different sponge species
32
33 588 (Diaz, 1979; Gaino et al., 1995; Korotkova, 1997; Ereskovsky et al., 2015; Borisenko et al.,
34
35 589 2015; Lavrov & Kosevich, 2016; Lavrov et al., 2018). For example, direct transdifferentiation
36
37 590 of cells in intact epithelia (pinacocytes and choanocytes) has been demonstrated during the
38
39 591 reparative regeneration of the homoscleromorph *Oscarella lobularis* (Ereskovsky et al.,
40
41 592 2015), the calcareous sponges *Sycon lingua* (Korotkova, Efremova, & Kadantseva, 1965;
42
43 593 Korotkova, 1972b), and *Leucosolenia* ssp. (Lavrov et al., 2018). In these cases, the layer of
44
45 594 choanocytes transdifferentiates into layer of pinacocytes without loss of epithelial structure,
46
47 595 without mesenchymal-to-epithelial transformation, and without contribution from cell
48
49 596 proliferation.

50 597 Cell transdifferentiation have also been described during reparative regeneration in all
51
52 598 investigated demosponges (for review see: Korotkova, 1997; Borisenko et al., 2015). While
53
54 599 archaeocytes directly differentiate into new cells, choanocytes and pinacocytes undergo
55
56 600 transdifferentiation to give rise for other cell types. It is characteristic, mainly, for
57
58 601 choanocytes from disintegrated choanocyte chambers, which can transdifferentiate, for
59
60 602 example, into exopinacocytes (Korotkova & Nikitin, 1969a,b; Borisenko et al., 2015).
603 603 Endopinacocytes in a less extent could [also transdifferentiate](#) into exopinacocytes (Thiney,

1
2
3 604 1972; Borisenko et al., 2015). The principal difference of the transdifferentiation in
4
5 605 demosponges in comparison with Homoscleromorpha + Calcarea lineage is an occurrence of
6
7 606 the disruption of epithelial layers and cell dedifferentiation prior to this process in the
8
9 607 demosponges.

10 608 The similar processes were observed during *A. cavernicola* regeneration. The
11
12 609 archaeocytes and dedifferentiated cells participate in the restoration of the lost structures in
13
14 610 this sponge. We identify two cells types, which undergo dedifferentiation during *A.*
15
16 611 *cavernicola* regeneration: choanocytes, spherulous cells, and population of anucleated cells of
17
18 612 mesohyl, including dedifferentiated pinacocytes, myocytes, and lophocytes. The choanocytes,
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20 613 despite their dedifferentiation and loss of the flagella and collars of microvilles, are well
21
22 614 distinguished on ultrathin sections due to the persistence of two centrioles in their apical part.
23
24 615 Anucleated amoebocytes differ from archaeocytes by a smaller nucleus without nucleolus and
25
26 616 the presence of small phagosomes. The dedifferentiation of the spherulous cells through a
27
28 617 complete or almost complete loss of specific inclusions (spherules) was completely
29
30 618 unexpected process. However, the dedifferentiation of spherulous cells is not so rapid, as in
31
32 619 other dedifferentiating cells, because of high specialization of these secretory cells.

33 620 We identified two main cellular sources for the restoration of the lost tissue and
34
35 621 structures in *A. cavernicola*: archaeocytes and choanocytes. Both cell types contribute to the
36
37 622 restoration of the exopinacoderm and choanocyte chambers. The restoration of the choanocyte
38
39 623 chambers is carried out mainly due to the redifferentiation of the choanocytes, and to lesser
40
41 624 extent due to the differentiation of archaeocytes.

42 625 However, the future of dedifferentiated anucleated amoebocytes and spherulous cells
43
44 626 could not be traced. This issue requires the search and use of a special marker of these cells. It
45
46 627 is possible that dedifferentiated pinacocytes and spherulous cells could also participate in the
47
48 628 formation of the new exopinacoderm.

49 629 According to last investigations in Porifera there are not only two types of adult stem
50
51 630 cells (ASC): archaeocytes and choanocytes (Funayama, 2018), but at least four, including
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53 631 pinacocytes and particular amoeboid vacuolar cells (Ereskovsky et al., 2015; Fierro-Constain
54
55 632 et al., 2017; Lavrov et al., 2018).

56 633 There are the differences in these ASC distributions among different sponge classes. In
57
58 634 Demospongiae the principal (pluripotent) ASC are archaeocytes and choanocytes, however
59
60 635 the experiments with their regeneration (including present data on *A. cavernicola*
636 regeneration) and dissociated cells aggregation demonstrated, that pinacocytes could also be
637 ASC (Korotkova & Nikitin, 1969a,b; Borisenko et al., 2015; Lavrov & Kosevich, 2016;

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2
3 638 Ereskovsky et al., 2016). In *Calcarea* archaeocytes absent and pluripotent ASC in these
4
5 639 sponges are choanocytes and pinacocytes (Korotkova, 1961a, 1962b; Korotkova &
6
7 640 Gelihovskaia, 1963; Lavrov et al., 2018). In Homoscleromorpha the pluripotent ASC are as
8
9 641 in *Calcarea* the choanocytes and pinacocytes, but also mesohyl amoeboid vacuolar cells
10 642 (Gaino et al., 1986; Ereskovsky, 2010; Ereskovsky et al., 2015; Fierro-Constain et al., 2017).
11
12 643

13 644 **5. Spherulous cells and their role in regeneration**

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15 645 Similarly, to intact tissues of *A. cavernicola* (Vacelet, 1967), two principal morphotypes
16
17 646 of spherulous cells occurs in the wound area of this sponge: larger cells with clear inclusions
18
19 647 and smaller ones with dense inclusions. In fact, these morphotypes represent different stages
20
21 648 of the ontogenesis of spherulous cells (Vacelet, 1967). According to Vacelet (1967) in intact
22
23 649 sponge “dense” small spherulous cells concentrate mostly in the ectosome and around big
24
25 650 exhalant canals, while “clear” larger ones are distributed in the mesohyl of endosome.

26 651 We have detected that in *A. cavernicola* the wound surface was free from invasive
27
28 652 microbes from the first hours after injury up to the end of the regeneration. **All microbes, that**
29
30 653 **we detected at the wound surface and in the wound area during regeneration, shows**
31
32 654 **ultrastructure identical to the microbes, located in the deeper sponge tissues and described as**
33
34 655 **symbiotic in the previous papers (Vacelet, 1975; Fiedrich et al., 1999; Hentschel et al., 2001).**
35
36 656 **Such defense against invasive microbes is probably provided by the particular chemical**
37
38 657 **substances in the spherulous cells.**

39 658 X-ray microanalysis revealed that these specialized cells of *Aplysina* produce and store
40
41 659 brominated metabolites (Turon et al., 2000). Turon et al. (2000) demonstrated that the
42
43 660 concentration of bromine peaks is slightly higher in the spherules from “dense” spherulous
44
45 661 cells than in “clear” ones. Thompson et al. (1983) have detected bromine in the spherulous
46
47 662 cells of Caribbean *Aplysina fistularis*. Spherulous cells have been shown to produce defense
48
49 663 metabolites in other species (Thompson et al., 1983; Bretting et al., 1983; Uriz et al., 1996a).

50 664 In addition, it was showed that tissue damage in *Aplysina* induces a bioconversion of
51
52 665 isoxazoline alkaloids into aeroplysinin-1 and secondary metabolite dienone (Thoms et al.,
53
54 666 2006). This reaction is likely catalyzed by enzymes, and it may be ecologically important as
55
56 667 the dienone has the strong antibacterial power and toxicity (Reverter et al., 2016). Moreover,
57
58 668 this injury-induced reaction takes place within less than 1 min after wounding (Thoms et al.,
59
60 669 2006).

60 671 **6. Apoptosis during *A. cavernicola* regeneration**

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2
3 672 We have directly visualized and investigated apoptosis in the regeneration of sponges
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5 673 for the first time. The only previous data on the involvement of apoptosis in sponge
6
7 674 regeneration emerge from the comparative transcriptomic analysis of the early stages of body
8
9 675 wall regeneration in *Halisarca caerulea* (Kenny et al., 2017).

10 676 We have observed two waves of apoptosis during *Aplysina cavernicola* regeneration.
11
12 677 The first wave, occurring around 6 hpo, is probably involved in the elimination of damaged
13
14 678 cells and is characteristic for the early stages of regeneration of various animals, from
15
16 679 cnidarians to vertebrates (Vlaskalin et al., 2004; Tseng et al., 2007; Chera et al., 2009;
17
18 680 Pellettiere et al., 2010; DuBuc et al., 2014; Brandshaw et al., 2015; Kenny et al., 2017; Cebrià
19
20 681 et al., 2018). For *Hydra* head regeneration after mid-gastric bisection (Chera et al., 2009) and
21
22 682 tail regeneration in tadpole larva of *Xenopus laevis* (Tseng et al., 2007) it was shown that
23
24 683 early wave of apoptosis not only clean a wound from damaged cells, but also generate signals,
25
26 684 essential for initiation of subsequent regenerative processes. Inhibition of this apoptotic wave
27
28 685 completely abolishes the regeneration. In particular, in both cases, apoptotic cells initiate a
29
30 686 synchronous burst of proliferative activity in neighboring cells (Tseng et al., 2007; Chera et
31
32 687 al., 2009), which is similar to the well-known apoptosis-induced compensatory proliferation
33
34 688 in the imaginal disks of *Drosophila* (Fan & Bergmann, 2008; Bergmann & Steller, 2010).
35
36 689 Apoptotic cells may be involved in the proliferation induction also during *A. cavernicola*
37
38 690 body wall regeneration, as numerous DNA-synthesizing cells appears in the blastema only
39
40 691 after the first apoptotic wave, at 24 hpo. These observations illustrate a novel active
41
42 692 instructing role of apoptosis in morphogenetic processes, in contrast to its canonical passive
43
44 693 role as a destructive agent (Duffy, 2012).

41 694 The second wave of apoptosis during *A. cavernicola* regeneration is likely associated
42
43 695 with the extensive thrown out of spherulous cells though the forming exopinacoderm.
44
45 696 Spherulous cells of *A. cavernicola* demonstrate the similar behavior in the intact sponges.
46
47 697 Being one of the few terminally differentiated sponge cell lines, spherulous cells leave the
48
49 698 mesohyl through aquiferous system canals or external surfaces (Vacelet, 1967). Their release
50
51 699 outside from sponge is part of their normal physiology and possibly involved in the release of
52
53 700 various chemicals (Uriz et al., 1996a; Ternon et al., 2016) and/or discharge processes
54
55 701 (Maldonado, 2016) to the environment. The ultrastructure of spherulous cells eliminated
56
57 702 through the exopinacoderm during the third phase of *A. cavernicola* regeneration is identical
58
59 703 to that of the spherulous cells at the last stage of their evolution (Vacelet, 1967). Similarly, in
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704 the intact tissues of *H. caerulea*, many caspase-3 positive cells have a spherulous cell
705 morphology (De Goeij et al., 2009), which indicates that apoptosis involved in the waste

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2
3 706 control system in sponges in addition to maintaining tissue homeostasis (De Goeij et al.,
4 707 2009).

5
6 708 Thus, the described increase in the level of apoptosis at the late stages of the *A.*
7 709 *cavernicola* regeneration could be referred as a general tissue response, involved in the
8 710 restoration of the normal sponge physiology in the area of injury, rather than in regeneration
9 711 process itself. In planarians, the similar late general apoptotic response is also described, but it
10 712 participates in the intensive remodeling of intact tissues, adjacent to a wound, to restore
11 713 proper scale and proportions (Pellettieri et al., 2010; Cebrià et al., 2018).

12 714

13 715 **Conclusion**

14 716 Finally, we can made some principal conclusions:

15 717 1. In Porifera there are two principal mode of reparative regeneration: blastemal
16 718 regeneration and tissue remodeling. From the literature data and results, obtained in this work
17 719 we can conclude that first mode is characteristic for Demospongiae regeneration and the
18 720 second – for Calcarea + Homoscleromorpha clade.

19 721 2. The results of our research also showed that sponges have more than two lines of
20 722 adult stem cells, the potencies of which are clearly manifested during reparative regeneration.
21 723 Our data support previous investigations, showed that archaeocytes and choanocytes are main
22 724 players, or principal adult stem cells during demosponges regeneration (Korotkova & Nikitin,
23 725 1969; Thiney, 1972; Boury-Esnault, 1976; Diaz, 1979; Borisenko et al. 2015). However, the
24 726 pinacocytes also could be considered as multipotent stem cells. Moreover, for the first time
25 727 we showed, that spherulous cells of *Aplysina cavernicola* have a capacity to dedifferentiation
26 728 during regeneration. It is first evidence that highly specialized cells of demosponges have
27 729 capacity to dedifferentiation and possibly transdifferentiation.

28 730 3. Cell transdifferentiation plays an extremely significant role in sponge regeneration, as
29 731 we have shown that regardless of the phylogenetic position, type of aquiferous system, and
30 732 structure of epithelia, all studied sponges intensively utilize it for restoration of lost structures
31 733 (Borisenko et al., 2015; Ereskovsky et al., 2015, 2017; Lavrov et al., 2018).

32 734 4. For the first time was investigated apoptosis during sponge regeneration. In
33 735 *Aplysina cavernicola* regeneration this processes participate in damaged cells elimination and
34 736 associated with the extensive ejection of spherulous cells from wound area.

35 737

36 738 **Author contributions**

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2
3 739 A.V.E. and A.I.L. designed the study, conducted the experimental procedures with the living
4 740 animals; A.I.L. performed the cell proliferation studies; A.V.E. and D.B.T. performed
5 741 histological and ultrastructural studies. A.V.E.: Project administration and funding
6 742 acquisition; S.B., E.L.G., and A.I.L.: performed the apoptosis studies; A.V.E. and A.I.L.
7 743 prepared the manuscript with contributions from all authors. All authors reviewed and
8 744 approved the final manuscript.
9 745

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761

1
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3 1020 **Figure legends for *Aplysina cavernicola* regeneration**

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6 1022 **Figure 1.** Time series photographs *in situ* of wounds to *Aplysina cavernicola*.

8 1023 Time series photographs of wounds to *Aplysina cavernicola*, Corrals Cave, Marseille, France
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10 1024 June-July 2018. Two examples were chosen to illustrate individual variety of regeneration
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12 1025 after experimental wounding. W – wound. Scale bar – 1.2 cm.

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15 1027 **Figure 2.** *Aplysina cavernicola in vivo*

17 1028 A – intact sponge before operation; B – sponge just after operation; C – sponge after 48 hours
18
19 1029 after operation.

20 1030 o – osculum, w – wound.

21
22 1031

23
24 1032 **Figure 3.** Intact *Aplysina cavernicola*

25 1033 A – semi-fin section of upper part of sponge; B – TEM image of ectosome; C –
26 1034 exopinacocyte and spherulous cells (inset: detail of cell junctions between exopinacocytes); D
27 1035 – endopinacocyte; E – choanocyte chamber (inset: choanocyte); F – pocket cell with
28
29 1036 symbiotic bacteria; G – archaeocyte; H – myocytes (inset: detail of myocytes with
30
31 1037 myofibrils).

34 1038 ar – archaeocyte, cb – cell body of exopinacocyte, cc – choanocyte chamber, ch – choanocyte,
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36 1039 ect – ectosome, en – endopinacocyte, end – endosome, ex- exopinacocytes, f – flagellum, fp –
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38 1040 flat part of exopinacocyte, in – inclusions in spherulous cells, my – myocyte, pc - pocket cell,
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40 1041 n – nucleus, sb – symbiotic bacteria, sc – spherulous cell.

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43 1043 **Figure 4.** Cells of the mesohyl in intact *Aplysina cavernicola*

44 1044 A – bacteriocyte; B – spongocyte; C – lophocytes; D - spherulous cell at the late stage of
45
46 1045 evolution expelled into exhalant canal; E – microgranular cell; F – granular cell; G, H –
47
48 1046 spherulous cells in two stages of their development.

49 1047 ar – archaeocyte, ba – bacteriocyte, ex- exopinacocytes, exc – exhalant canal, in – inclusions
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51 1048 in spherulous cells, lo – lophocyte, mc – microgranular cell, n – nucleus, sb – symbiotic
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53 1049 bacteria, sc – spherulous cell, sf – skeleton fiber, sp - spongocyte.

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56 1051 **Figure 5.** Scheme of wound and their parts in *Aplysina cavernicola*.

58 1052 ect – ectosome, end – endosome, it - intact tissues, ra – regenerated area, w – wound, wa –
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60 1053 wound area, we – wound edge.

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Figure 6. 3h of regeneration in *Aplysina cavernicola*

A – wound and a marginal zone; B – zone under the wound; C – internal part of the wound with encapsulated invasive diatoms; D – dedifferentiated choanocyte (flash show the basal flagellum apparatus)

ar – archaeocyte, ba – bacteriocyte, dch – dedifferentiated choanocyte, di – encapsulated diatom, en – endopinacocyte, in – inclusions in spherulous cells, lo – lophocyte, n – nucleus, ph – phagosome, sb – symbiotic bacteria, sc – spherulous cell, scf – spherulous cells fragments, w - wound.

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Figure 7. 6h of regeneration in *Aplysina cavernicola*

A – semi thin section of the wound and upper part of endosome; B, C – TEM of the upper part of the wound: it is a choanocyte chamber during their destruction, fragments of spherulous cells, phagocyted spherulous cell; D, E – wound zone with the fragments of cells and phagocytosis of spherulous cells; F – inner part of the wound, degraded choanocyte chamber.

ar – archaeocyte, cc – choanocyte chamber, dcc - destructed choanocyte chamber, dch – dedifferentiated choanocyte, end – endosome, in – inclusions in spherulous cells, n – nucleus, ph – phagosome, sb – symbiotic bacteria, sc – spherulous cell, scf – spherulous cells fragments, w – wound, ws – wound surface.

1074

Figure 8. Boxplot diagram of the number of spherulous cells per mm² in intact tissues and during regeneration in *A. cavernicola*. The differences between some of the means are statistically significant (ANOVA p-value = 2.36E-07(<0.01)). Tukey's multiple comparisons showed no significant differences only in 3 pairs: Intact tissues–96 hpo; 6 hpo–12 hpo; 24 hpo–48 hpo. Duncan's new multiple range test (MRT) approve this, grouping these pairs into three clusters (A, B, C). The differences between Duncan's clusters are statistically significant (Supporting Table 4).

1082

Figure 9. 12h of regeneration in *Aplysina cavernicola*

A – semi thin section of the wound and upper part of endosome; B, C – TEM of the upper part of the wound, there are some non-secret cells at the wound surface (arrowheads), dedifferentiated choanocytes and spherulous cells; D – detail of the upper part of the wound;

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3 1087 E – wound zone with dedifferentiated choanocytes; F – marginal zone of the wound with the
4 1088 part of intact exopinacocyte.

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6 1089 ar – archaeocyte, cf – cells fragments, dch – dedifferentiated choanocyte, dsc -
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8 1090 dedifferentiated spherulous cell, end – endosome, in – inclusions in spherulous cells, n –
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10 1091 nucleus, ph – phagosome, sb – symbiotic bacteria, sc – spherulous cell, scf – spherulous cells
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12 1092 fragments, w – wound, ws – wound surface.

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15 1094 **Figure 10. 24h of regeneration in *Aplysina cavernicola***

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17 1095 A – semi thin section of the wound and upper part of endosome; B, C – TEM of the upper
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19 1096 part of the wound, with flat parts of new exopinacocytes, spherulous and non-secret cells with
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21 1097 the phagosomes (arrowhead - basal body of choanocyte flagellum); D – wound zone with
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23 1098 dedifferentiated choanocytes; **E - upper part of the wound zone with archaeocyte and**
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25 1099 **dedifferentiated choanocyte in the beginning of differentiation into new exopinacocyte, arrow**
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27 1100 **show the basal apparatus of dedifferentiated choanocyte; F - archaeocyte differentiated into**
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29 1101 **exopinacocyte at the upper part of the wound.**

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31 1102 ar – archaeocyte, cf – cells fragments, dch – dedifferentiated choanocyte, dsc -
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33 1103 dedifferentiated spherulous cell, end – endosome, in – inclusions in spherulous cells, n –
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35 1104 nucleus, **nu – nucleolus**, ph – phagosome, sb – symbiotic bacteria, sc – spherulous cell, scf –
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37 1105 spherulous cells fragments, w - wound.

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38 1107 **Figure 11. 48h of regeneration in *Aplysina cavernicola***

39 1108 A – semi thin section of the wound and upper part of endosome; B – TEM of the upper part of
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41 1109 the wound, with restored ectosome; C – TEM of the upper part of the wound, showed
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43 1110 transdifferentiation of a choanocyte into exopinacocyte; **inset – basal apparatus of**
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45 1111 **dedifferentiated choanocyte (arrowhead); D – TEM of the upper part of the wound, showing**
46
47 1112 **transdifferentiation of an archaeocyte into exopinacocyte; E, F - TEM of the small apoptotic**
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49 1113 **spherulous cells elimination of apoptotic spherulous cells through forming exopinacoderm.**

50 1114 ac – apoptic cell, asc – apoptic spherulous cell, ar – archaeocyte, cc – choanocyte chamber, ch
51
52 1115 – choanocyte, cf – cells fragments, dch – dedifferentiated choanocyte, end – endosome, ex-
53
54 1116 exopinacocytes, fp – flat part of exopinacocyte, in – inclusions in spherulous cells, lo –
55
56 1117 lophocyte, mc – microgranular cell, my – myocyte, n – nucleus, ph – phagosome, sb –
57
58 1118 symbiotic bacteria, sc – spherulous cell, scf – spherulous cells fragments, w – wound zone.

58 1119

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60 1120 **Figure 12. 48h of endosome regeneration in *Aplysina cavernicola***

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3 1121 A-C – TEM of the endosome under the wound, showing different stages of restoration of
 4 1122 exhalant canals of the aquiferous system; D-F - TEM of the endosome under the wound,
 5 1123 showing different stages of restoration of choanocyte chambers.

6 1124 cc – choanocyte chamber, ch – choanocyte, en – endopinacocyte, exc – exhalant canal, sb –
 7 1125 symbiotic bacteria.

8 1126

9 1127 **Figure 13. 96h of regeneration in *Aplysina cavernicola***

10 1128 A – semi thin section of the completely restored ectosome and upper part of endosome; B –
 11 1129 TEM of the upper part of the restored ectosome; C - the endosome with new choanocyte
 12 1130 chamber; D – the endosome with normal cell composition.

13 1131 ar – archaeocyte, cc – choanocyte chamber, ch – choanocyte, ect – ectosome, end –
 14 1132 endosome, exc- exhalant canal, lo – lophocyte, n – nucleus, sb – symbiotic bacteria, sc –
 15 1133 spherulous cell.

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17 1135 **Figure 14. Cell proliferation in intact tissues of *Aplysina cavernicola*.** A – ectosome; B –
 18 1136 endosome; C – endosome near the exhalant canal of the aquiferous system; D – DNA-
 19 1137 synthesizing choanocytes with small round to oval nuclei; E – mesohyl DNA-
 20 1138 synthesizing cells with large round nuclei. Cyan – DAPI, green – α -tubulin, magenta –
 21 1139 EdU. cc – choanocyte chamber, end – endosome, ect – ectosome, exc – exhalant canal of the
 22 1140 aquiferous system. White arrowheads marks EdU-positive nuclei of DNA-synthesizing cells.
 23 1141 Each image is a maximum projection, obtained from 55 μ m Z stack.

24 1142

25 1143 **Figure 15. Cell proliferation during *Aplysina cavernicola* body wall regeneration.** A-E –
 26 1144 cell proliferation in the wound area at different stages of regeneration: A – 0-6 hpo; B – 0-24
 27 1145 hpo; C – 24-48 hpo; D – 48-72 hpo; E – 120-144 hpo; F – cell proliferation in intact tissues,
 28 1146 distant from the wound, during regeneration. Cyan – DAPI, magenta – EdU. Dcc –
 29 1147 disintegrating choanocyte chamber, cc – choanocyte chamber. White arrowheads marks EdU-
 30 1148 positive nuclei of DNA-synthesizing cells. Each image is a maximum projection, obtained
 31 1149 from 15 μ m Z stack.

32 1150

33 1151 **Figure 16. Apoptosis during *Aplysina cavernicola* body wall regeneration.** A – 6 hpo; B –
 34 1152 12 hpo; C – 48 hpo. Blue – DAPI, red – TUNEL. White dashed line marks the wound edge,
 35 1153 white arrows – TUNEL-positive nuclei of apoptotic cells. Each image is an optical slice
 36 1154 through the wound area.

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5 1156 **Supporting Figure 1. TEM images of ECM dynamic at the wound surface.**

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7 1158 A – intact ; B – 0 hpo ; C - 3 hpo ; D – 6 hpo ; E – 12 hpo ; F – 24 hpo ; G – 48 hpo ; H – 96
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9 1159 hpo.
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For Peer Review

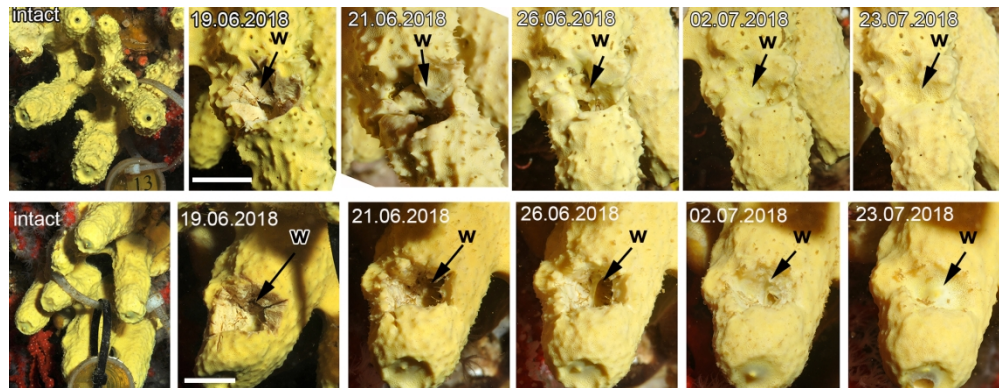


Figure 1. Time series photographs in situ of wounds to *Aplysina cavernicola*.
Time series photographs of wounds to *Aplysina cavernicola*, Corrals Cave, Marseille, France June-July 2018.
Two examples were chosen to illustrate individual variety of regeneration after experimental wounding. W –
wound. Scale bar – 1.2 cm.

179x68mm (300 x 300 DPI)

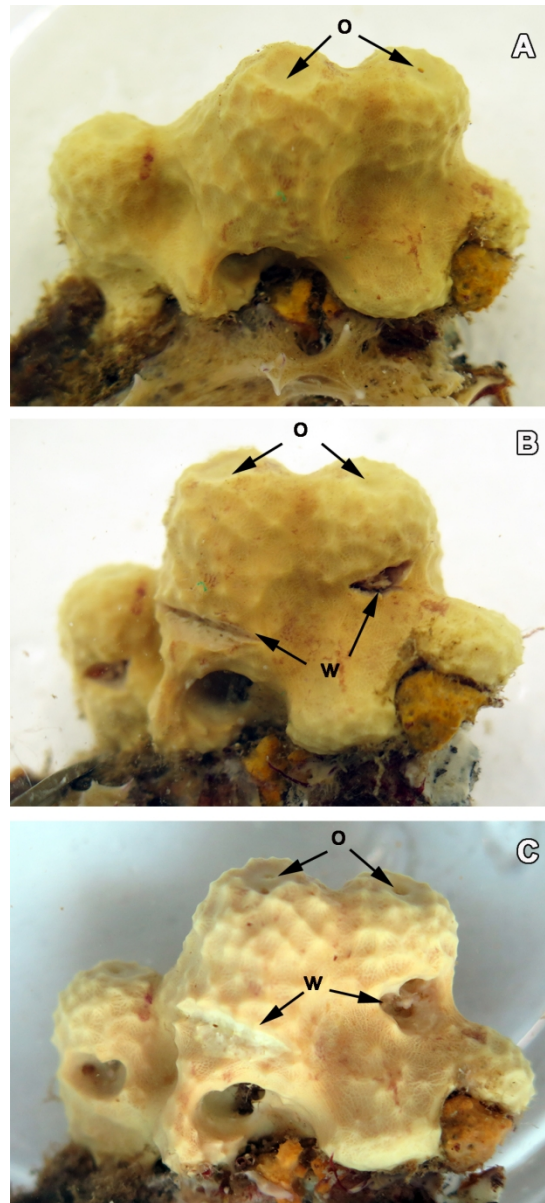


Figure 2. *Aplysina cavernicola* in vivo
A – intact sponge before operation; B – sponge just after operation; C – sponge after 48 hours after operation.

o – osculum, w – wound.

90x198mm (300 x 300 DPI)

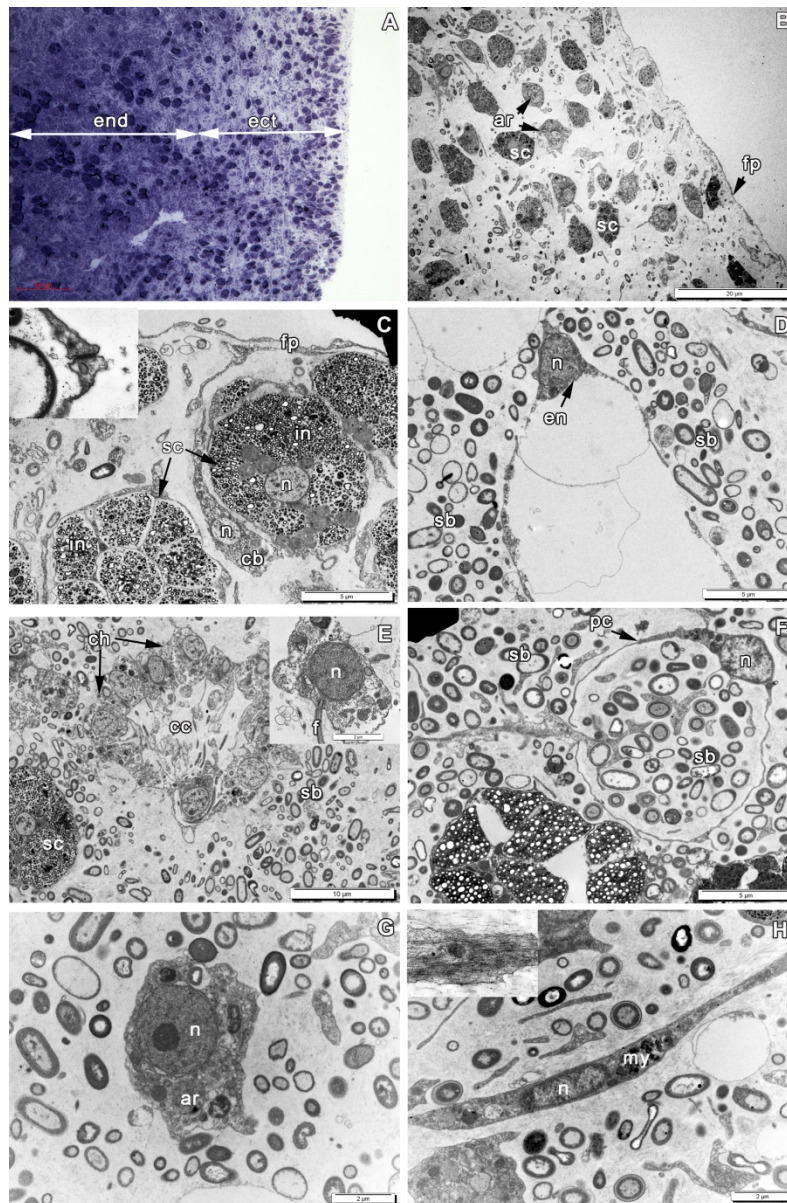


Figure 3. Intact *Aplysina cavernicola*

A – semi-fine section of upper part of sponge; B – TEM image of ectosome; C – exopinacocyte and spherulous cells (inset: detail of cell junctions between exopinacocytes); D – endopinacocyte; E – choanocyte chamber (inset: choanocyte); F – pocket cell with symbiotic bacteria; G – archaeocyte; H – myocytes (inset: detail of myocytes with myofibrils).
 ar – archaeocyte, cb – cell body of exopinacocyte, cc – choanocyte chamber, ch – choanocyte, ect – ectosome, en – endopinacocyte, end – endosome, ex – exopinacocytes, f – flagellum, fp – flat part of exopinacocyte, in – inclusions in spherulous cells, my – myocyte, pc – pocket cell, n – nucleus, sb – symbiotic bacteria, sc – spherulous cell.

180x272mm (300 x 300 DPI)

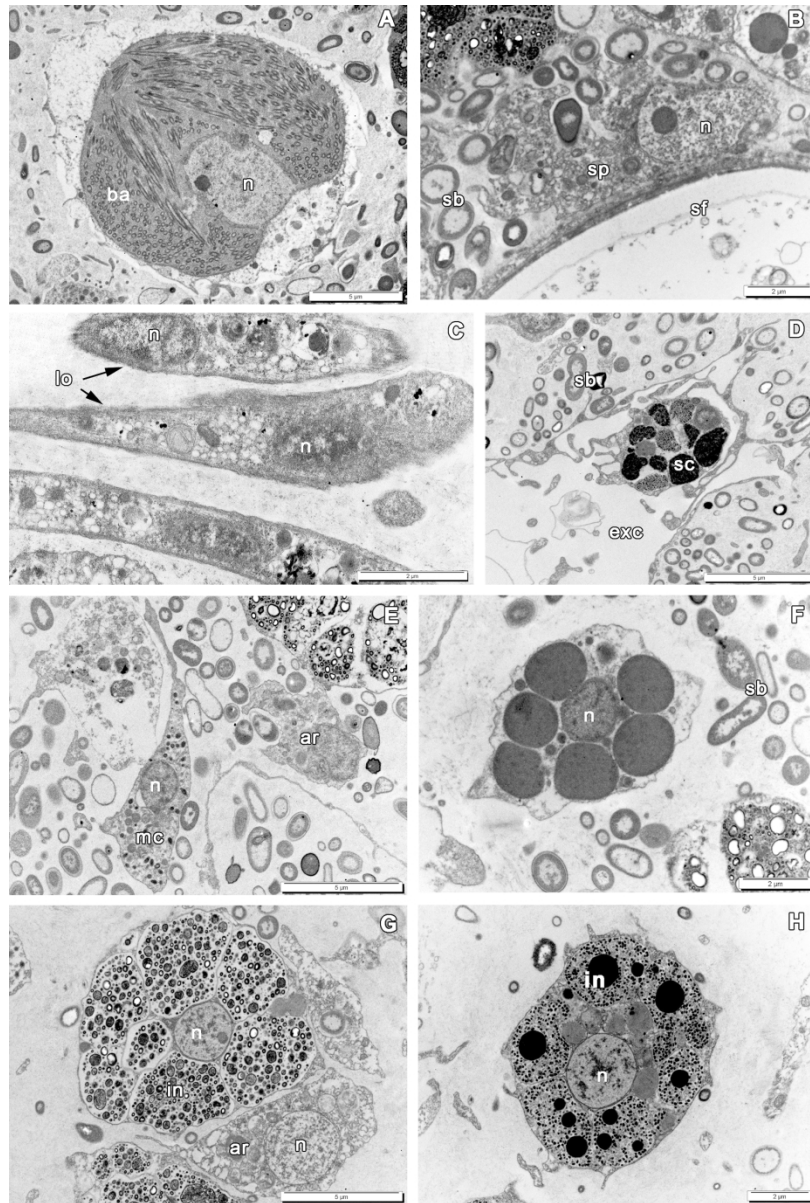


Figure 4. Cells of the mesohyl in intact *Aplysina cavernicola*

A – bacteriocyte; B – spongocyte; C – lophocytes; D - spherulous cell at the late stage of evolution expelled into exhalant canal; E – microgranular cell; F – granular cell; G, H – spherulous cells in two stages of their development.

ar – archaeocyte, ba – bacteriocyte, ex- exopinacocytes, exc – exhalant canal, in – inclusions in spherulous cells, lo – lophocyte, mc – microgranular cell, n – nucleus, sb – symbiotic bacteria, sc – spherulous cell, sf – skeleton fiber, sp - spongocyte.

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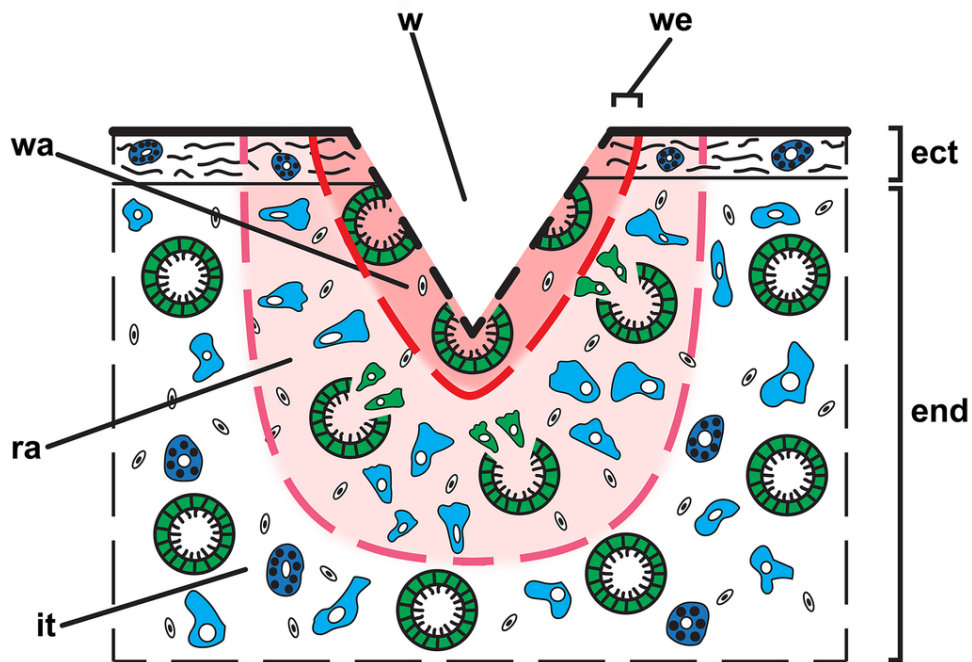


Figure 5. Scheme of wound and their parts in *Aplysina cavernicola*.

ect – ectosome, end – endosome, it - intact tissues, ra – regenerated area, w – wound, wa – wound area, we – wound edge.

90x68mm (300 x 300 DPI)

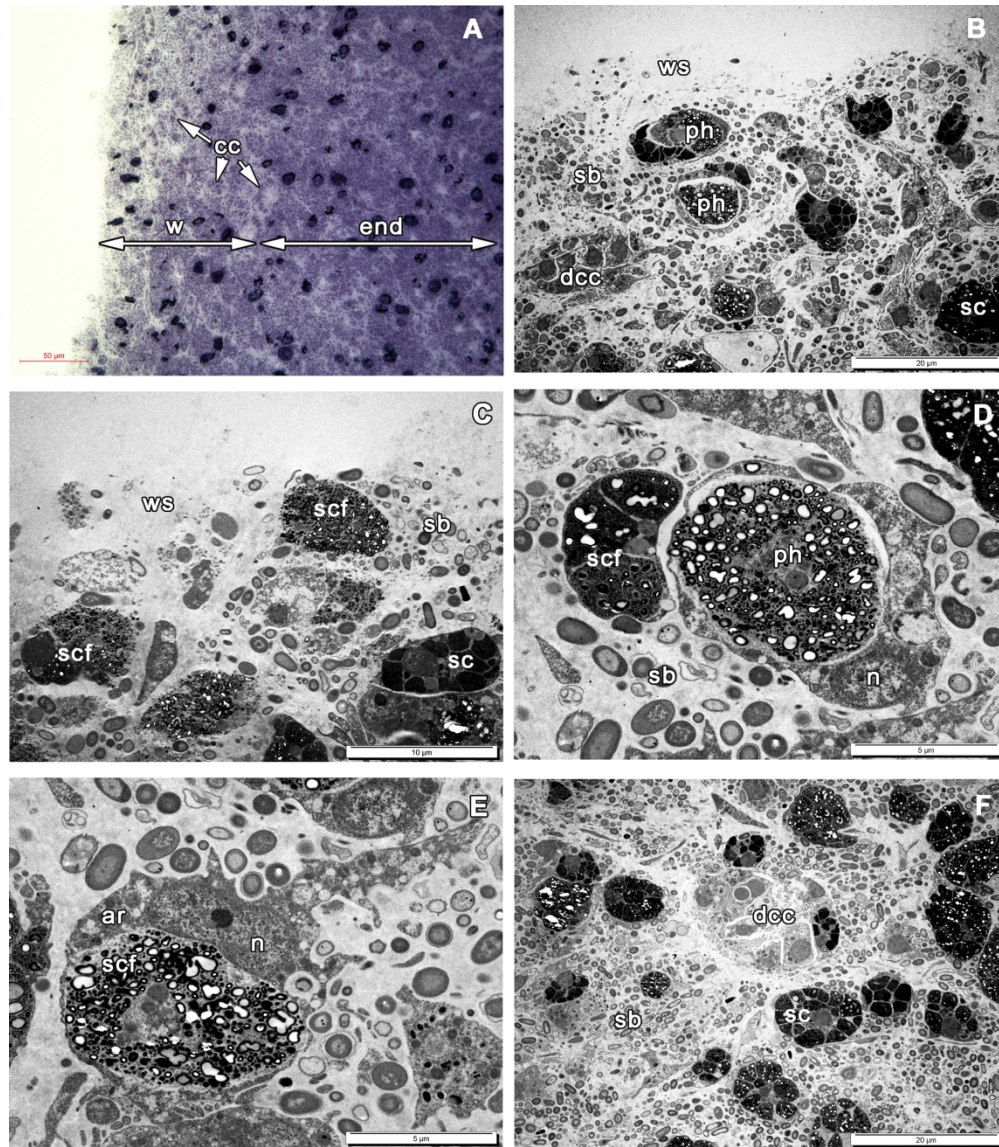


Figure 7. 6h of regeneration in *Aplysina cavernicola*

A – semi thin section of the wound and upper part of endosome; B, C – TEM of the upper part of the wound: it is a choanocyte chamber during their destruction, fragments of spherulous cells, phagocytosed spherulous cell; D, E – wound zone with the fragments of cells and phagocytosis of spherulous cells; F – inner part of the wound, degraded choanocyte chamber.

ar – archaeocyte, cc – choanocyte chamber, dcc - destructed choanocyte chamber, dch – dedifferentiated choanocyte, end – endosome, in – inclusions in spherulous cells, n – nucleus, ph – phagosome, sb – symbiotic bacteria, sc – spherulous cell, scf – spherulous cells fragments, w – wound, ws – wound surface.

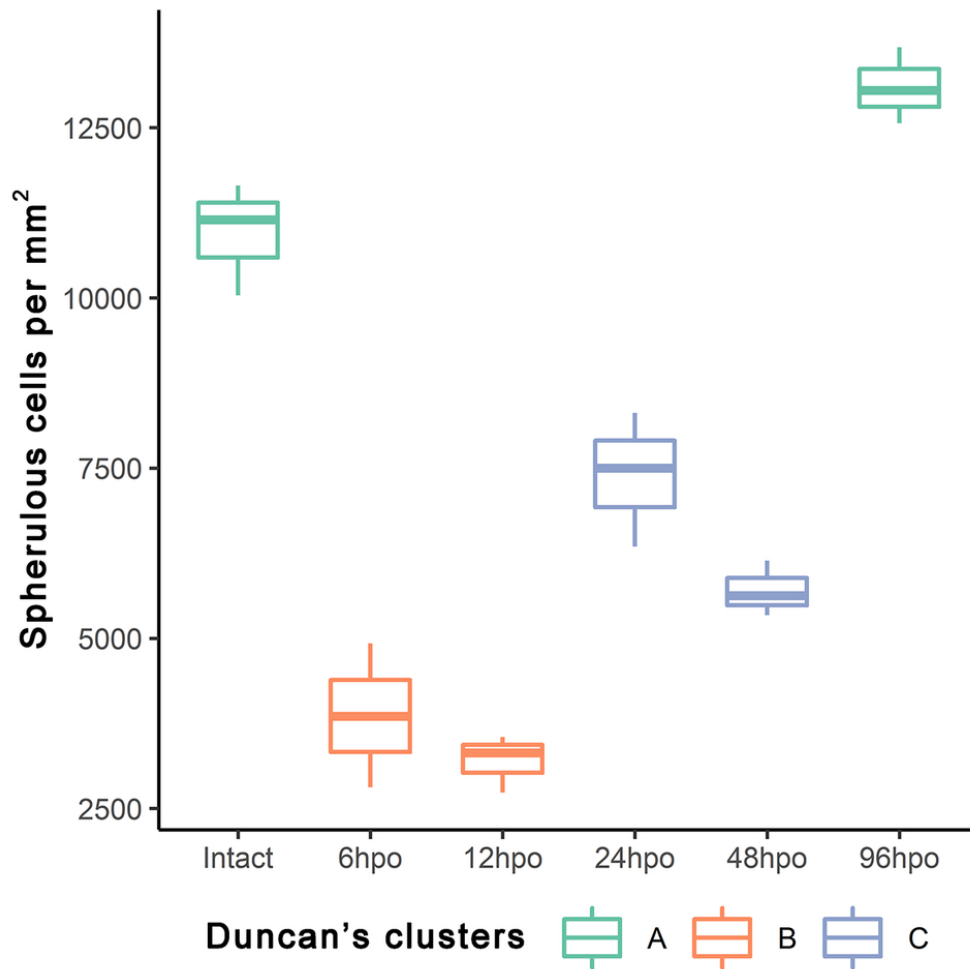


Figure 8. Boxplot diagram of the number of spherulous cells per mm² in intact tissues and during regeneration in *A. cavernicola*. The differences between some of the means are statistically significant (ANOVA p-value = 2.36E-07(<0.01)). Tukey's multiple comparisons showed no significant differences only in 3 pairs: Intact tissues–96 hpo; 6 hpo–12 hpo; 24 hpo–48 hpo. Duncan's new multiple range test (MRT) approve this, grouping these pairs into three clusters (A, B, C). The differences between Duncan's clusters are statistically significant (Supporting Table 4).

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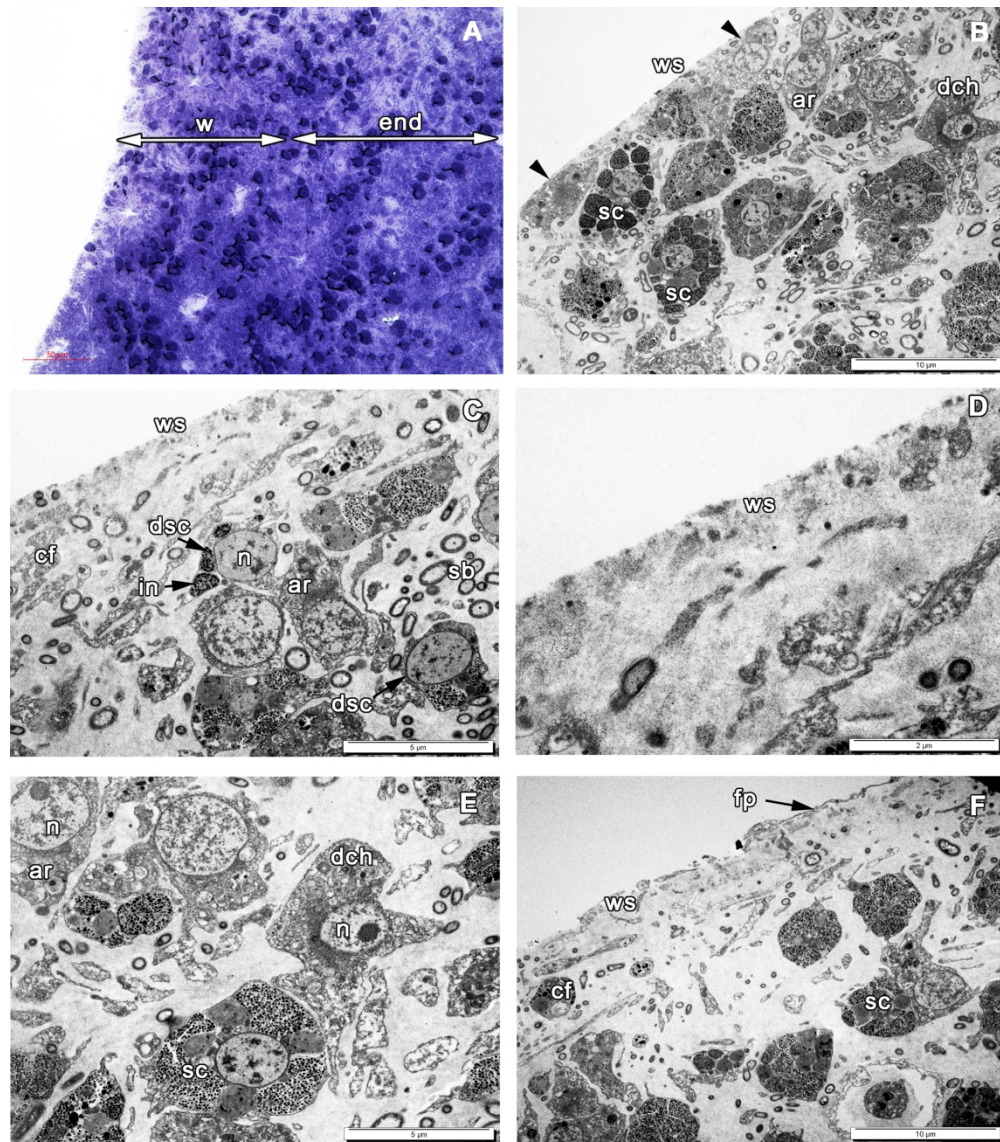


Figure 9. 12h of regeneration in *Aplysina cavernicola*

A – semi thin section of the wound and upper part of endosome; B, C – TEM of the upper part of the wound, there are some non-secret cells at the wound surface (arrowheads), dedifferentiated choanocytes and spherulous cells; D – detail of the upper part of the wound; E – wound zone with dedifferentiated choanocytes; F – marginal zone of the wound with the part of intact exopinacocyte.
 ar – archaeocyte, cf – cells fragments, dch – dedifferentiated choanocyte, dsc – dedifferentiated spherulous cell, end – endosome, in – inclusions in spherulous cells, n – nucleus, ph – phagosome, sb – symbiotic bacteria, sc – spherulous cell, scf – spherulous cells fragments, w – wound, ws – wound surface.

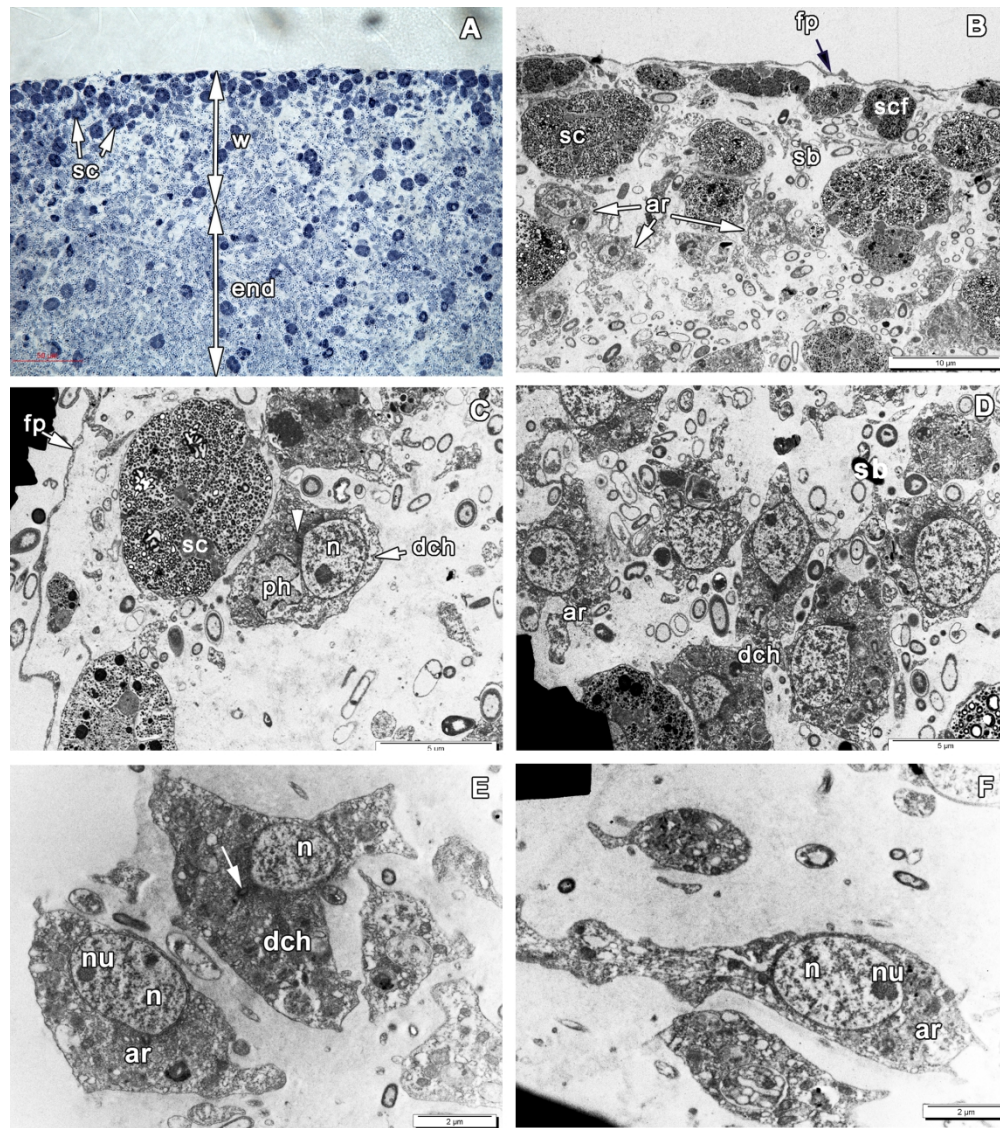


Figure 10. 24h of regeneration in *Aplysia cavernicola*

A – semi thin section of the wound and upper part of endosome; B, C – TEM of the upper part of the wound, with flat parts of new exopinacocytes, spherulous and non-secret cells with the phagosomes (arrowhead - basal body of choanocyte flagellum); D – wound zone with dedifferentiated choanocytes; E - upper part of the wound zone with archaeocyte and dedifferentiated choanocyte in the beginning of differentiation into new exopinacocyte, arrow show the basal apparatus of dedifferentiated choanocyte; F - archaeocyte differentiated into exopinacocyte at the upper part of the wound.
 ar – archaeocyte, cf – cells fragments, dch – dedifferentiated choanocyte, dsc - dedifferentiated spherulous cell, end – endosome, in – inclusions in spherulous cells, n – nucleus, nu – nucleolus, ph – phagosome, sb – symbiotic bacteria, sc – spherulous cell, scf – spherulous cells fragments, w - wound.

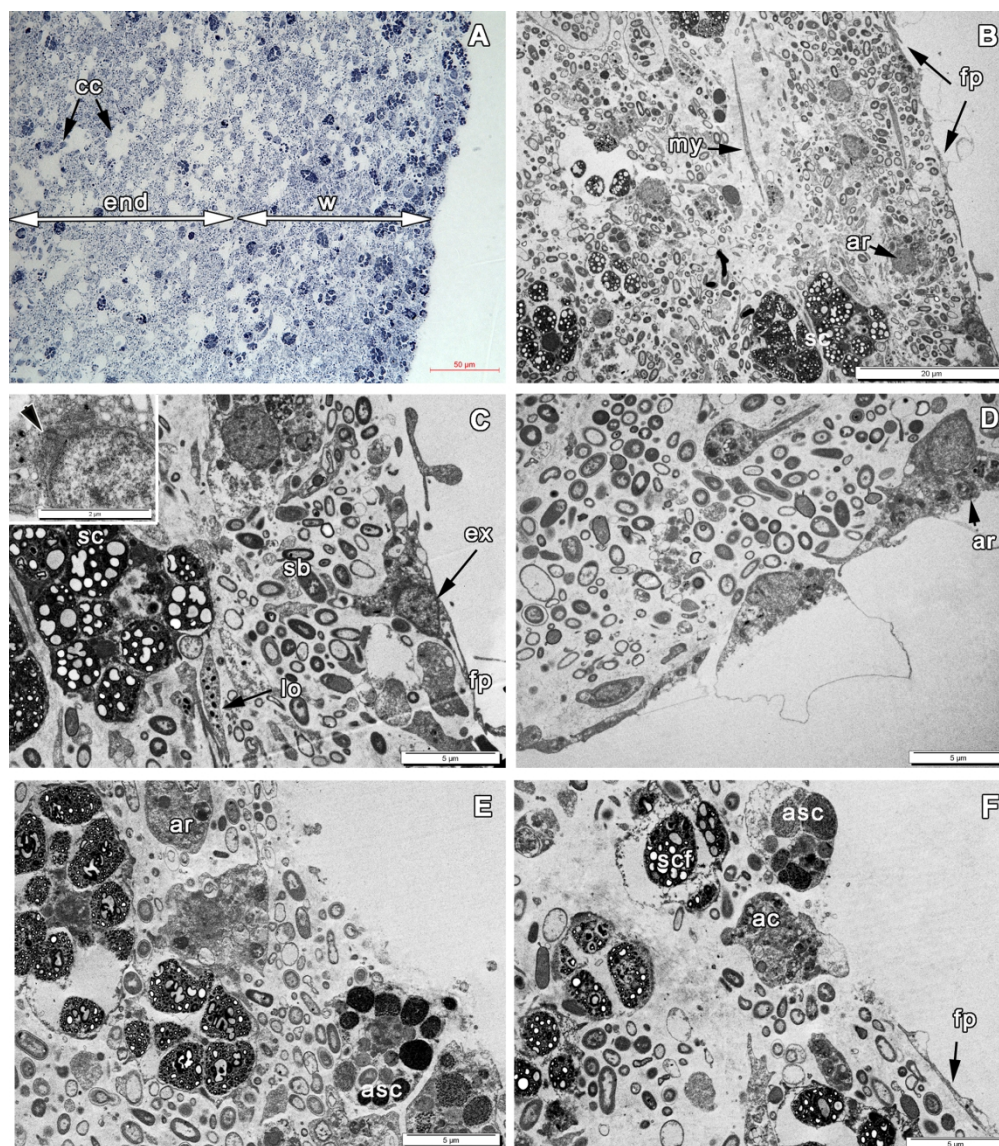


Figure 11. 48h of regeneration in *Aplysina cavernicola*

A – semi thin section of the wound and upper part of endosome; B – TEM of the upper part of the wound, with restored ectosome; C – TEM of the upper part of the wound, showed transdifferentiation of a choanocyte into exopinacocyte; inset – basal apparatus of dedifferentiated choanocyte (arrowhead); D – TEM of the upper part of the wound, showing transdifferentiation of an archaeocyte into exopinacocyte; E, F – TEM of the small apoptotic spherulous cells elimination of apoptotic spherulous cells through forming exopinacoderm.

ac – apoptic cell, asc – apoptic spherulous cell, ar – archaeocyte, cc – choanocyte chamber, ch – choanocyte, cf – cells fragments, dch – dedifferentiated choanocyte, end – endosome, ex- exopinacocytes, fp – flat part of exopinacocyte, in – inclusions in spherulous cells, lo – lophocyte, mc – microgranular cell, my – myocyte, n – nucleus, ph – phagosome, sb – symbiotic bacteria, sc – spherulous cell, scf – spherulous cells fragments, w – wound zone.

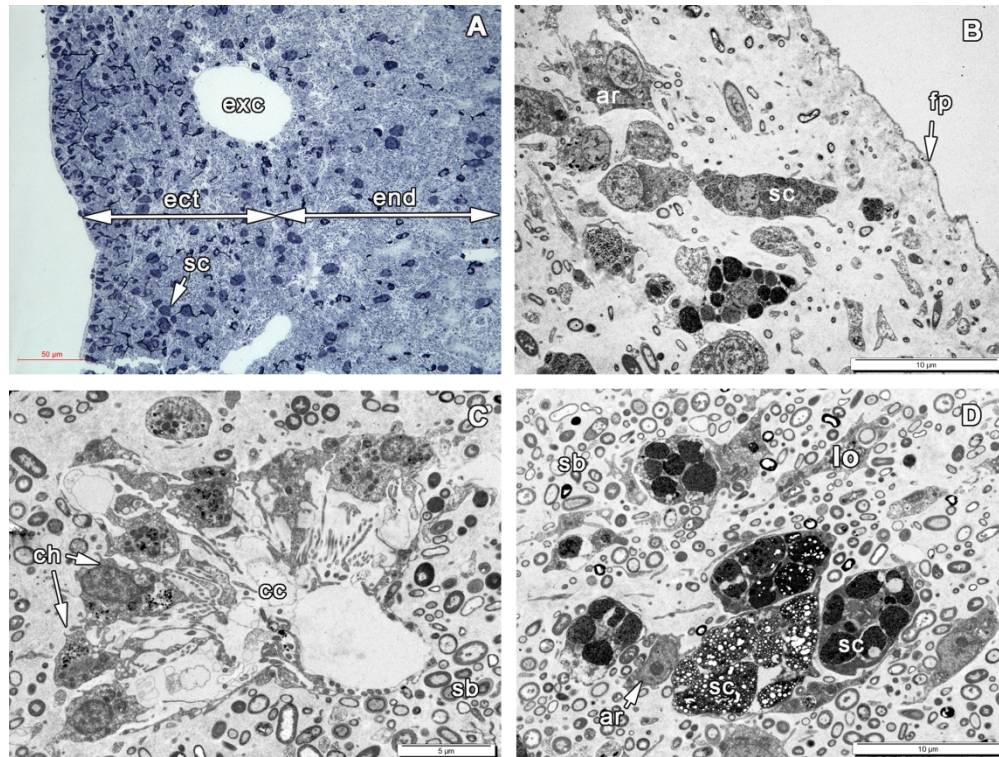


Figure 13. 96h of regeneration in *Aplysina cavernicola*

A – semi thin section of the completely restored ectosome and upper part of endosome; B – TEM of the upper part of the restored ectosome; C - the endosome with new choanocyte chamber; D – the endosome with normal cell composition.

ar – archaeocyte, cc – choanocyte chamber, ch – choanocyte, ect – ectosome, end – endosome, exc – exhalant canal, lo – lophocyte, n – nucleus, sb – symbiotic bacteria, sc – spherulous cell.

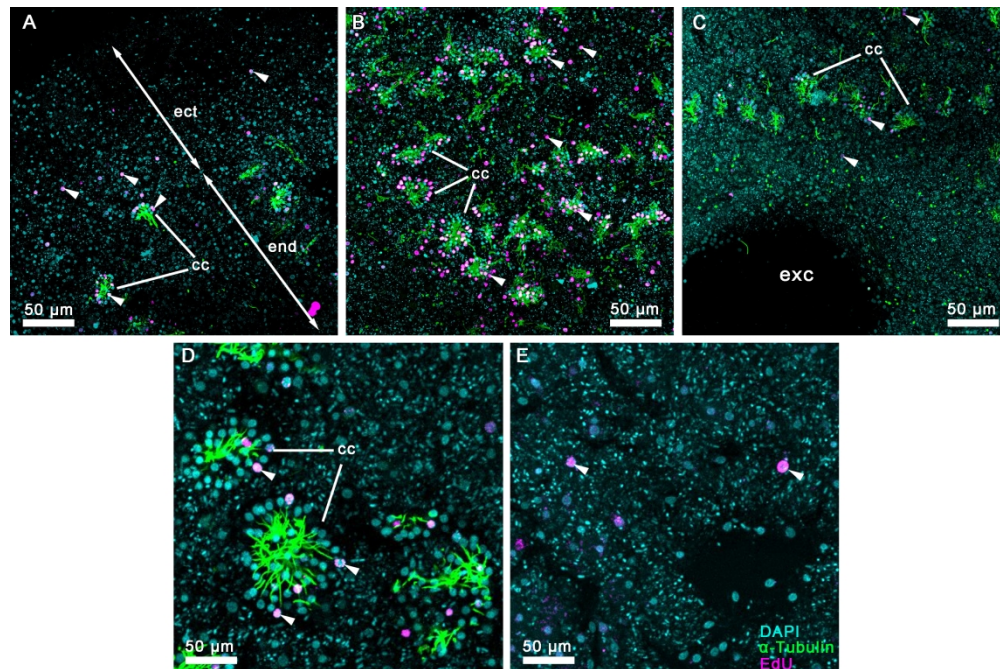
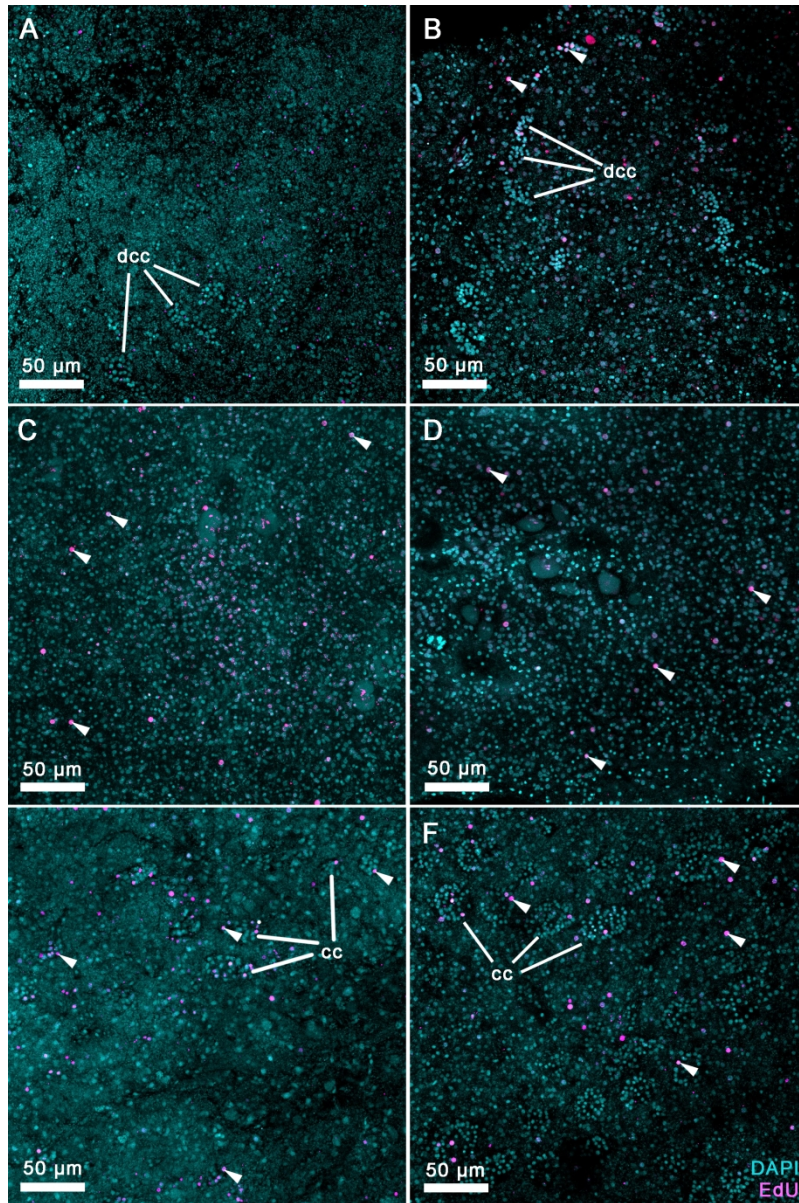


Figure 14. Cell proliferation in intact tissues of *Aplysina cavernicola*. A – ectosome; B – endosome; C – endosome near the exhalant canal of the aquiferous system; D – DNA-synthesizing choanocytes with small round to oval nuclei; E – mesohyl DNA-synthesizing cells with large round nuclei. Cyan – DAPI, green – α -tubulin, magenta – EdU. cc – choanocyte chamber, end – endosome, ect – ectosome, exc – exhalant canal of the aquiferous system. White arrowheads marks EdU-positive nuclei of DNA-synthesizing cells. Each image is a maximum projection, obtained from 55 μ m Z stack



45 Figure 15. Cell proliferation during *Aplysina cavernicola* body wall regeneration. A-E – cell proliferation in the
 46 wound area at different stages of regeneration: A – 0-6 hpo; B – 0-24 hpo; C – 24-48 hpo; D – 48-72 hpo;
 47 E – 120-144 hpo; F – cell proliferation in intact tissues, distant from the wound, during regeneration. Cyan –
 48 DAPI, magenta – EdU. dcc – disintegrating choanocyte chamber, cc – choanocyte chamber. White
 49 arrowheads marks EdU-positive nuclei of DNA-synthesizing cells. Each image is a maximum projection,
 50 obtained from 15 µm Z stack.

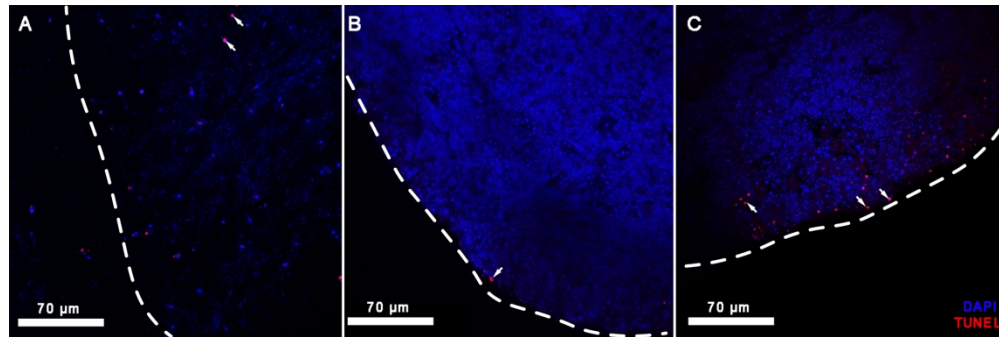


Figure 16. Apoptosis during *Aplysina cavernicola* body wall regeneration. A – 6 hpo; B – 12 hpo; C – 48 hpo. Blue – DAPI, red – TUNEL. White dashed line marks the wound edge, white arrows – TUNEL-positive nuclei of apoptotic cells. Each image is an optical slice through the wound area.