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The hypothetical ancestral animal. the Urmetazoa: telomerase activity in sponges (Porifera)*

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Abstract: Sponges (Porifera) represent the lowest metazoan phylum, characterized by a pronounced plasticity in the determination of cell lineages, and they are the closest related taxon to the hypothetical ancestral animal, the Urmetazoa, from which the metazoan lineages diverged. In a first approach to elucidate the molecular mechanisms controlling the switch from the cell lineage with a putative indefinite growth capacity to senescent, somatic cells, the activity of the telomerase as an indicator for immortality has been determined. The studies were performed with the marine demosponges *Suberites domuncula* and *Geodia cydonium*, *in vivo* with tissue but also *in vitro* using the primmorph system. Primmorphs are formed from dissociated cells which have retained their proliferation potency. It was found that the activity of telomerase in tissue of both sponges is high. Based on this and additional findings it is assumed that the separation of the senescent sponge cell lineage from the immortal germ-/somatic cell lineage is triggered by the loss of contact to cell adhesion factors. First evidence is included which suggests that the final progress of the senescent, telomerase-negative cells to cell death is caused by apoptosis.

Keywords: *Suberites domuncula*, *Geodia cydonium*, primmorphs, senescence, telomeres, telomerase, cell lineages, Urmetazoa.

INTRODUCTION: URMETAZOA - HYPOTHETICAL ANCESTOR OF METAZOA

Sponges (Porifera) represent the simplest multicellular animals. With respect to body organization, cell differentiation and embryogenesis sponges have been considered as being separated from other metazoans and have been grouped in Parazoa or Archaemetazoa (review)[1], both in traditional animal classifications such as proposed by Hyman[2] and Barnes,[3] and in some modern molecular studies on animal evolution.[4] Another phylum, the Placozoa, has been classified together with the sponges into the subkingdom Metazoa, to separate them from the second animal subkingdom, the Eumetazoa.[5]

* Dedicated to Professor M. Gašić in commemoration of his profound and longstanding achievements in organic natural compounds.

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However, recent molecular data, obtained by sequence analyses of nucleic acids, cDNAs and proteins have not given support to the separation of Porifera from Eumetazoa, thus implying that all metazoans are of monophyletic origin[6–9] and originate from the hypothetical ancestor, the Urmetazoa.[10] This conclusion is largely based on sequence data of several genes/cDNAs obtained from the marine demosponges *Geodia cydonium* and *Suberites domuncula*, coding for adhesion molecules (galectin) and adhesion receptors (integrin receptor, receptor(s) featuring scavenger-receptor cysteine-rich domains), or elements involved in signal transduction pathways (tyrosine kinase receptors, G-proteins, Ser/Thr protein kinases), see reviews.[11,12] The presence of common molecular mechanisms for both structural and metabolic cell integration in sponges on one side and in higher metazoans on the other side, points towards their close evolutionary relationship and a common route of development of multicellularity in the animal kingdom. Therefore, we proposed that all metazoan phyla including the Porifera evolved from a common ancestor, the hypothetical Urmetazoa.[9,13]

During the transition from unicellular Protoctista to multicellular Metazoa, the primary pattern of differentiation implies the presence of at least two different cell types; the simplest multicellular organism could consist of one cell type specialized for feeding and the other for reproduction.[14,15] This is in agreement with the original Roux-Weismann's concept of primary separation of somatic and germinal cell lineages,[16] in which the immortal germen produces a mortal soma, that will sustain the growth and reproduction of the organism but will necessarily perish. The concept of programmed senescence and death of the somatic cell lineage is consequently inherent to the concept of early separation of the soma and the germ-cell lineages.[17] In view of the proposed monophyletic evolution of Metazoans and the position of sponges at the base of the evolution of multicellularity, we have addressed the question for those molecular mechanisms which underlie the evolution of the germ cell- and somatic cell lineages, and the potential control of their immortality or their programmed senescence and death.[18]

The origin of the germ cells in sponges is still debated. The vast majority of studies on spermatogenesis report the development of spermatids from spermatocysts and of spermatogonia, originating directly from functional choanocyte chambers.[19,20] The origin of oogonia is less clear: they were reported to derive from choanacytes in *Suberites massa*,[21] but most authors recognize the precursors of eggs among the mesohyl cells with the archaeocyte morphology.[22,23] The definition and identification of putative stem cells for primordial germ cells in sponges have not been clearly provided, and the compelling morphological evidence for the origin of gametes from the somatic fully differentiated cells, such as choanocytes, argues against the clear separation of the germinal and somatic cell lineages.

Two major theories have been presented to explain cellular ageing: the theory of terminal differentiation and the one of genetic instabilities (see reviews[24,25]). Recently, ageing has been associated with the telomeric DNA shortening of chromosomes: it was found experimentally that the length of telomeric DNA in human fibroblasts decreases as a func-

tion of serial passage during ageing *in vitro* and possibly also *in vivo*.[26] This hypothesis is attractive because the process of loss of telomeric DNA is restricted to somatic cells in higher Metazoa (see review[27]), while cells with unlimited replicative potential, such as reproductive cells[28] or immortal tumor cells[29] have stable telomeres.

The polymerase which mediates the synthesis of additional telomeric repeats was first identified in HeLa cells and was termed telomerase.[30] The activity of that enzyme correlates positively with the extent of telomere addition in a given tissue.[27] Hence, the level of telomerase activity in a given cell population may be used as a parameter to determine its immortality. In order to elucidate whether sponge cells display characteristics of the postnatal somatic tissues observed in higher metazoan phyla, in which the reduction in telomerase activity is associated with the programmed senescence and mortality of somatic cell lineages, the activity of this enzyme was determined in the tissue from *S. domuncula* and *G. cydonium*. The results reveal indeed that high levels of telomerase are present in those animals.

PRIMMORPHS: MODEL SYSTEM TO STUDY THE DIFFERENTIATION STATE OF SPONGE CELLS

Establishment of primmorph culture

Our group has focused on the formation of primmorphs[31] from the demosponges *S. domuncula*,[32,33] *Dysidea avara*[34] and occasionally *G. cydonium*. *S. domuncula* occurs in nature in red, orange, whitish, blue or as a mixture of these colors (Fig. 1a) while tissue samples from 20 – 60 cm large *G. cydonium* animals are yellowish or grey (Fig. 1b). The procedure of primmorph formation, with respect to *S. domuncula*, is briefly outlined. Tissue samples are transferred into CMFSW-E (Ca^{2+} and Mg^{2+} -free artificial seawater containing EDTA). After shaking for 90 min, the supernatant, containing the dissociated single cells (Fig. 1c) is collected by centrifugation. The cells in the final pellets are resuspended in seawater, supplemented with antibiotics, to a density of 1.5–2.0 $\times 10^6$ cells/ml and the cultures are kept at 16 ºC. Immediately after transfer to the Ca^{2+} and Mg^{2+} -containing seawater, the single cells form small, 20 cells containing aggregates (Fig. 1d) which enlarge in size during the subsequent 12 to 24 h to 150 µm (Fig. 1e) and 1,000 µm large (Fig. 1f) cell clumps. After usually five days primmorphs are formed (Fig. 1g).

Characteristics – cell proliferation. The primmorphs are characterized by the presence of proliferating cells as well as by a characteristic histology. The BrdU (5-bromo-2'-deoxy-uridine)-labeling and detection assay was used to demonstrate that the cells organized in the primmorphs regain the capacity to proliferate. The BrdU-positive cells, undergoing DNA synthesis, are stained brownish in their nuclei (Fig. 1i). the percentage of BrdU-positive cells present in cell aggregates formed from single cells after one day in culture is low; only 6.5 % are counted to be positive. In contrast, the number of DNA-synthesizing/proliferating cells present in primmorphs is high and reaches values of 20 % to 30 % depending on the age of the primmorphs (Fig. 1j).

Characteristics – histology. The diameter of the cell aggregates increases steadily after an incubation period of approximately three days (Fig. 1f). After a total treatment/incu-

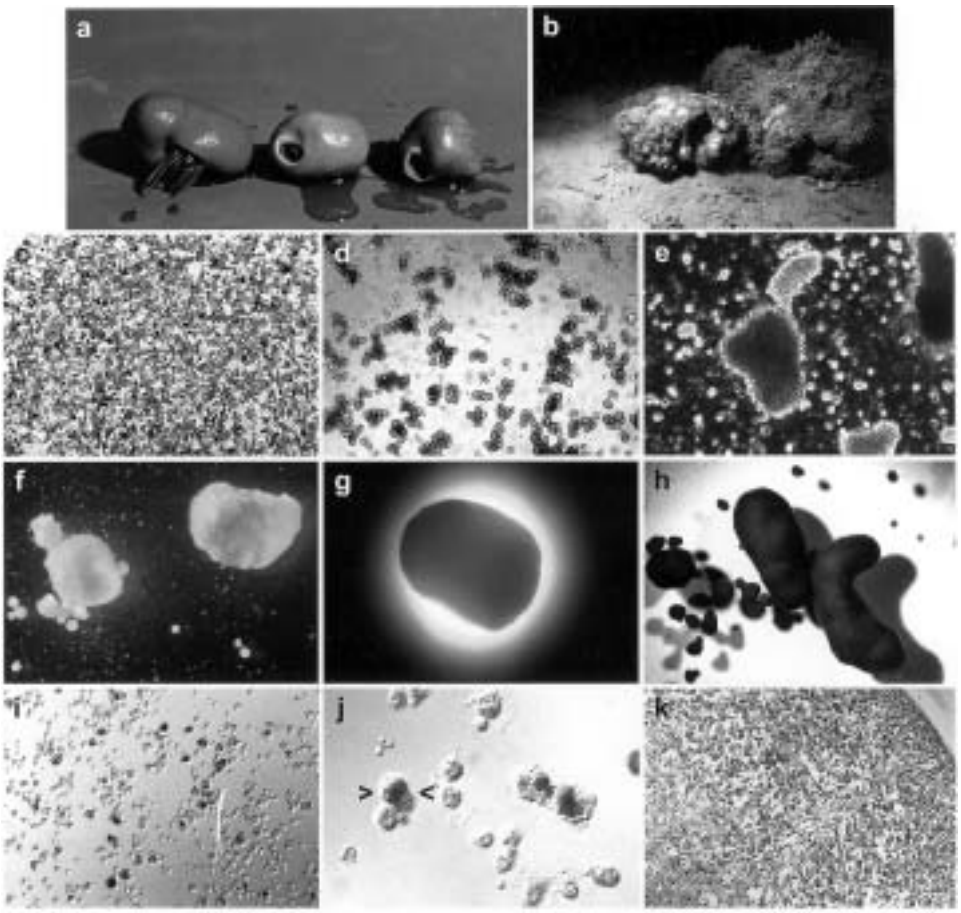

Fig. 1. Primmorph formation from cells of the sponges *S. domuncula* and *G. cydonium.* (a) *S. domuncula* occurs in red, orange, whitish or blue or as a mixture [magnification; × 0.5]. (b) A specimen of *G. cydonium* [× 0.05]. Primmorph formation from cells of *S. domuncula*: Dissociated single cells [× 10]; (c). After transfer to seawater aggregates are formed which range from a size of 30 μm (d) after 6 h, to 150 μm (e) after 12 h to 1,000 μm (f); [× 10]. Primmorphs formed after 5 days (g); [× 8]. (h) Primmorphs formed after five days in the presence of recombinant myotrophin (1 μg/ml); [× 5]. DNA synthesis in primmorphs was determined using the BrdU-labeling and detection assay. After incubation of the primmorphs with BrdU the cells were dissociated and subjected to staining with anti-BrdU monoclonal antibody. The dark brownish stained nuclei are those which have incorporated BrdU (i and j); [× 50 and × 250]. In j the arrows mark one BrdU-positive cell. Cross sections through one primmorph show that the interior is surrounded by an almost complete single-cellular layer of epithelial-like cells (k); [× 50].

bation period of about five days, primmorphs are formed from cell aggregates (Fig. 1g). During the phase of primmorph formation the aggregates contract to round-shaped bodies, 1 to 5 mm in size, leaving behind detritus and dead cells. In the initial phase the primmorphs remain round-shaped but after incubation of longer than three to four weeks many of them attach to the bottom of the culture dish.

Cross sections through the primmorphs were performed. Microscopic analysis of the sections stained with Ziehl's fuchsin revealed that the cells present in the interior are surrounded by an almost complete single-cellular layer of epithelial-like cells (Fig. 1k). The cells which compose the squamous epithelium of the primmorphs are pinacocytes as judged from their flattened, fusiform extensions and their prominent nucelus. The cells inside the primmorphs are primarily spherulous cells while the others may be termed amoebocytes and archaeocytes. The organized arrangement of the cells within the primmorphs distinguishes them from aggregates which are formed from dissociated cells in the presence of the homologous aggregation factor.

Growth conditions

Chemical factors. The growth conditions could (until now) be optimized by supplementing the natural seawater with 0.2 % of RPMI1640-medium and silicate. The optimal concentration of silicate was determined to be 60 μm.[35]

One growth promoting protein has been isolated from *S. domuncula* which was shown to stimulate proliferation of sponge cells; the myotrophin-like polypeptide.[36] The cDNA of the sponge myotrophin was isolated; the potential open reading frame of 360 nt encodes a 120 aa long protein with a calculated M_r of 12,837. The sequence shares high similarity with the known metazoan myotrophin sequences. The sponge sequence shows the characteristic features known from the vertebrate myotrophins[37]; one half of an ankyrin repeat is located at the NH_2-terminus of the protein (aa_{10} to aa_{28}) followed by two complete repeats towards the COOH-terminus (aa_{30} to aa_{62} and aa_{63} to aa_{95}). The expression of sponge myotrophin is low in single cells but is strongly upregulated after formation of primmorphs as well as in intact animals.[36]

Recombinant sponge myotrophin was prepared and found to stimulate overall protein synthesis by 5-fold.[36] Additionally, it was shown that after incubation of single cells with myotrophin the primmorphs show an elongated, oval-shaped appearance (Fig. 1h). In a successful attempt it was demonstrated that in the presence of recombinant myotrophin the cells upregulate the expression of the collagen gene. For these studies the cDNA for *S. domuncula* collagen was isolated; the deduced aa sequence shows that the collagenous internal domain is rather short with only 24 G-x-y collagen triplets. Based on these data it is concluded that the sponge myotrophin causes in the homologous cells the same/similar effect like the cardiac myotrophin in mammalian cells, where it is involved in initiation of cardial ventricular hypertrophy. In addition, it is shown that myotrophin causes in primmorphs an increase in size and also a stimulation of macromolecular synthesis.

Myotrophin was the first growth factor isolated from sponges. At present, we are involved in the isolation and identification of a further factor which resulted in an increase of the diameter of the sponge primmorphs. While the size of the primmorphs grown in the absence of additional silicate is ≈ 2 mm, primmorphs which grew in the medium supplemented with 60 μM silicate reached sizes of 6 mm; a further supplementation of the sea-

water medium containing 60 μM silicate together with the not yet completely identified growth factor resulted in the formation of aggregates of a size of ≈ 10 mm.

TELOMERASE ACTIVITY IN PRIMMORPHS OF *S. domuncula*

As reported earlier,[18] and also summarized here, sponge cells undergo a transition from the telomerase-positive to a telomerase-negative state after dissociation into a single-cell suspension. To estimate if the level of telomerase activity is restored in cells during formation of primmorphs from a single-cell suspension, the activity was measured in cells after formation of primmorphs from *S. domuncula*.[32]

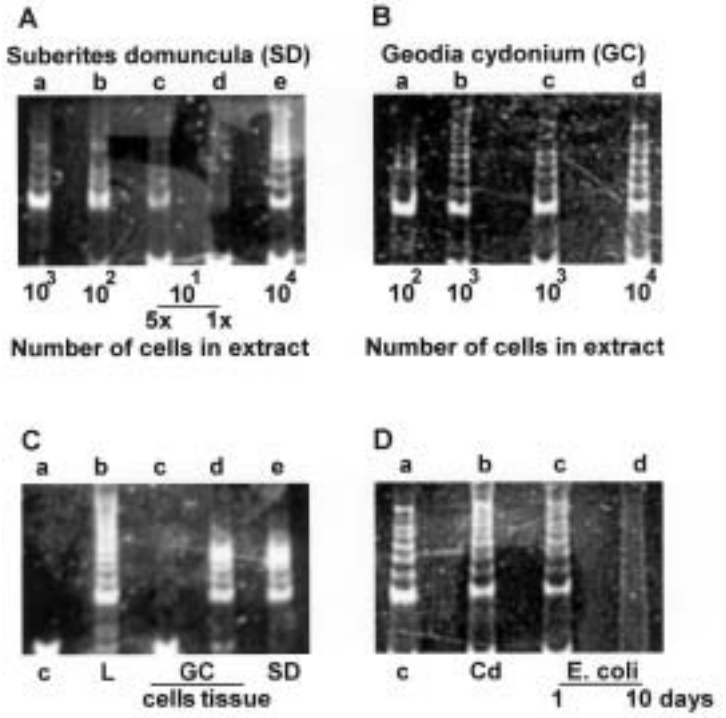

Fig. 2. Telomerase activity is sponge (*S. domuncula*) cells and tissue. The activity was determined in cells present in tissue (lane **b**), in the dissociated single-cell state - the cells have been analyzed 14 h after dissociation - (lanes **a** and **c**) and in primmorphs (lanes **d** and **e**). Defined amounts of tissue, corresponding to 5×10^3 cell equivalents, were assayed. After PCR amplification the products were resolved in a non-denaturing polyacrylamide gel and the gels were stained with SYBR Green I to detect the DNA fragments. "IC" is the internal control in the assay. The products of the telomerase reaction are visualized as a ladder of oligonucleotides with 6 base increments starting at 50 nucleotides: 50 (marked), 56, 62, 68, *etc*.[18]

As shown, cells in natural tissue are associated with high levels of telomerase activity; a quantitative analysis revealed an activity of 8.9 TPG units/5×10^3 cell equivalents (Fig. 2; lane b). In cells which had been left for 14 h in the dissociated single-cell state, the enzyme level dropped to < 0.9 TPG units/5×10^3 cells (Fig. 2; lanes a and c). However, in cells

from primmorphs (used 10 days after formation from single cells) a telomerase activity of 4.7 TPG units/5 × 10^3 cells is seen (Fig. 2; lane d). In comparison, if primmorphs which were cultivated for the same period of time either in the current chamber or in the aquarium, under conditions of strong water current,[33] the telomerase activity level is with 2.1 TPG units/5 × 10^3 cells lower. As outlined above primmorphs which are kept in the current chamber or under strong water current in aquarium express the homeodomain containing Iroquois transcription factor, which can be considered as a differentiation molecule.

These results confirm that cells if removed from the tissue assembly lose their telomerase activity. As shown already earlier,[18,38] single cells will recover after formation of tissue-like bodies, primmorphs, and turn from the telomerase-negative to the telomerase-positive state. In addition, the data show that primmorphs which contain cells undergoing potential differentiation (expression of Iroquois) display a reduced telomerase activity.

CONTROL OF CELL HOMEOSTASIS IN SPONGES: APOPTOSIS

It is assumed that some sponge species, *e.g.*, *G. cydonium*, are long living. The presented studies indicate that the somatic cells in tissue from the two demosponges, *G. cydonium* and *S. domuncula*, contain high levels of telomerase activity, a feature which is unique in the metazoan kingdom.[18] In higher Metazoa the telomerase can only be detected in cells of the germ line[28] and in immortal tumor cells,[29] while somatic cells are devoid of this enzyme; consequently they have only a limited potential for proliferation. It can therefore be deduced that the somatic cells of sponges have an unlimited proliferation- and differentiation potency.[18] However, the Bauplan of sponges is like in all metazoans a closed one, requiring a control mechanism which allows cell homeostasis, in a sense that the net cell number as the result of cell proliferation and cell death, is tunely balanced. The process which allows and guarantees such a balance is termed programmed cell death, or apoptosis (see review[39]).

Two forms of inducers of apoptosis must be distinguished.[40] Firstly, the intrinsic signals, both physiological inhibitors, *e.g.*, estrogens or androgens and physiological activators, like neutrotransmitters or calcium; and secondly, extrinsic signals, which can be again inhibitory (viral infection) or activating (heat shock) in their effect on the organism (see review[39]). In a first rational approach to determine if environmental hazardous compounds cause apoptosis in sponges, the marine sponge *G. cydonium* was used. Previously, it was described for the first time for sponges in particular and for Metazoa in general that xenobiotics, *e.g.*, tributyltin and methyl mercury, cause apoptosis.[41]

CONCLUSION

Based on the two observations that (*i*) the determination of germ- and somatic cell lineages is plastic in sponges and (*ii*) most sponge species show a continuous growth and a long life span, we addressed the question of the degree of immortality of cells in specimens of the lowest metazoan phylum, the Porifera. The telomerase activity was chosen as a pa-

rameter to estimate the level of immortality. It was found that the overall activity of the telomerase in sponge tissue is high. A quantitative analysis revealed that tissues from both *S. domuncula* and *G. cydonium* contained the activity of approximately 30 % and 20 %, respectively, of the telomerase activity in the positive reference cells.

In adult higher animals, telomerase activity is high in germ cells and in malignant tumors. A low activity can be detected in somatic stem cells, in tissues whose renewal depends upon extensive proliferation of its cells, such as the hematopoietic system, the bulb-containing fragment of the hair follicles, some epithelia, and germinative centers of lymph nodes.[42–44] In these somatic stem cells, this low telomerase activity is transient and responsive to growth factors.[45] Conversely, in immortal and maturation-sensitive cell lines, the induction of quiescence by either contact inhibition, growth factor removal or induction of terminal differentiation, the activity of telomerase is repressed.[46,47] In the studied sponges, the number of germ cells in tissues was very low or nil, and the observed high telomerase activity may indicate either that somatic sponge cells maintain the telomerase activity and have an unlimited replication potency, or the sponge tissues contain a large number of somatic stem cells able of unlimited proliferation, undergoing subsequently the terminal differentiation. In Cnidaria, a diploblastic group related to the Porifera, three self-renewing stem cell lineages with unlimited capacity of proliferation are in steady state, and can produce all the differentiated somatic cells, which are continuously shed from the tentacles or included in the growing buds.[48] In sponges, archaeocytes are considered to be pluripotent, equivalent to stem cells, and potentially produce all the major cell lineages, but morphological data indicate that the tissue homeostasis is normally maintained by proliferation of all major cell types.[49,50] The plasticity of the sex determination in sponges and the ability of fully differentiated cells such as choanocytes to give rise to gametes favor the hypothesis of the potentially high permanent telomerase activity in sponge somatic cells.

Since sponges are metazoans with integrated multicellular organization, they should be provided with mechanisms to control both the rate of cell proliferation and the rate of terminal differentiation or cell death, in order to allow a maintenance of homeostasis of the relative number of different cell types in tissues as well as of the absolute number of cells in a given specimen. From earlier studies it is known that single cells from *G. cydonium* that lack any contact to extracellular adhesion factors stop cell proliferation.[51] Telomerase activity is repressed in cells that exit the cell cycle,[47] and our observation that isolated sponge cells in suspension have undetectable level of telomerase activity is in compass with the cell growth inhibition. This is distinct from the fully transformed and maturation-resistant human cancer cells, which have no anchorage dependence and maintain both proliferation and telomerase activity in suspension.[46]

Taken together, the presented data indicate that somatic sponge cells have a high telomerase activity, which may be controlled by extrinsic factors such as contact with other cells or extracellular matrix. Sponge cell proliferation is under control of growth factors, *e.g.*, myotrophin, and the required major pathways of both tyrosine and serine/threonine kinases intracellular signaling have been described.[52–54] It will be in-

teresting to find whether these pathways may stimulate and maintain the high telomerase activity in somatic cells.

Alternatively, morphogen-like molecules such as retinoids have been shown to have specific activity on sponge cells,[55,56] and they may negatively regulate the telomerase activity inducing differentiation of sponge cells, similar to their activity on cells of the hematopoietic system.[45] Such a differentiation stimulatory factor is physical water current which, as shown here, causes the expression of the Iroquois homeodomain containing transcription factor.

Finally, concomitant regulation of apoptosis and telomerase activity have been proposed.[57] Apoptosis has been demonstrated in sponges, and sponge cells might undergo terminal senescence by activation of central death signals, *e.g.* activation of the expression of the MA-3 gene.[38] From an earlier study it is known that the activation of this gene results in the induction of programmed cell death (apoptosis).[58]

The apparently broad positive and negative regulation of telomerase activity in sponge cells indicates that they may be a useful model to study the molecular mechanisms of senescence controls. It has recently been summarized[59] that somatic cell growth arrest in Metazoa might be controlled by a complex mechanism driven by multiple senescence

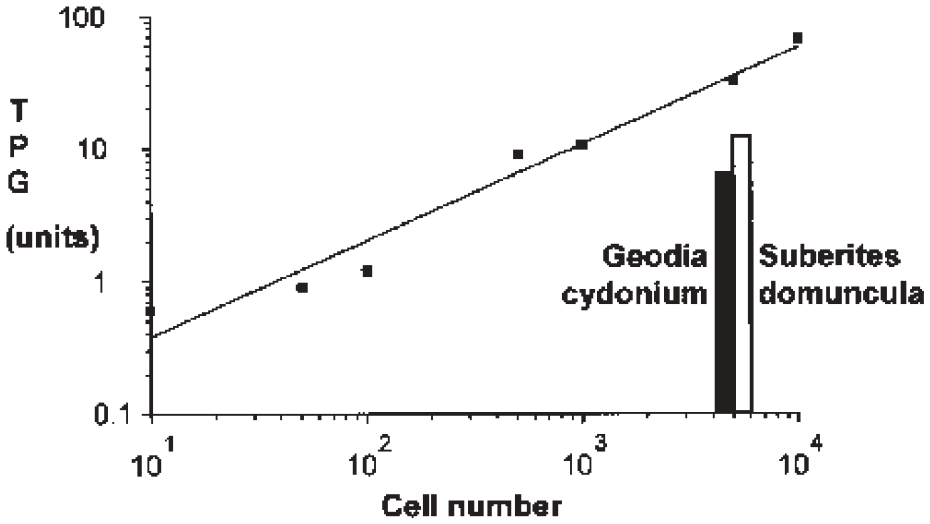

Fig. 3. Hypothetical determinants of immortality in species from higher Metazoa and in sponges. The lack of telomerase activity, and in consequence telomere loss, in somatic cells of higher metazoans determines their fate to senescence (circles) via the two phases: the "Mortality Phase 1" (M-1) - cell cycle arrest - and after transformation "Mortality Phase 2" (M-2). Cells of the germ lineage from higher Metazoa remain telomerase-positive and are immortal. In sponges (squares), it is proposed that the switch from immortal "somatic" cells, present in tissue and tissue like assemblies (primmorphs) to mortal cells (in the single cell stage) is triggered by both external and internal programs. The mortal cells are eliminated by the process of apoptosis which is controlled by both and pro- and anti-apoptotic programs. During the process of apoptosis in sponge cells, the expression of the gene *SDLAGL*, encoding the putative longevity assurance-like polypeptide, becomes downregulated (adapted from Refs. 18, 60).

pathways. According to Harley,[60,61] somatic cell senescence can be subdivided into two phases (Fig. 3). The "Mortality Phase 1" (M-1) leads cells to a permanent cell cycle senescence arrest, a checkpoint at which cells do not respond to any growth factor stimulation. In a second process, the telomeres of the "precrisis" cells reach a critical length, the cells enter "Mortality Phase 2" (M-2) and are prone to the signal for cell death.

A series of questions remain open. One major point is the elucidation of whether sponges and hence the hypothetical ancestor of the Metazoa, the Urmetazoa, have the clock that initiates the switch from immortal sponge cells to "somatic cells" committed to differentiation and designated to undergo senescence. Furthermore, the question has to be addressed whether and during which phase the reduction of telomeres in the terminal restriction fragments [TRFs] in sponge chromosomes occurs, and whether this reduction concerns a predetermined cell subpopulation, irreversibly commited to differentiation, such as observed in the hematopoietic system, or whether it is stochastic and potentially reversible under specific signals, such as induction of gametogenesis. Perhaps it might turn out that the program to differentiate the immortal stem cells to germ and somatic cells is a novelty restricted to the animal phyla younger in evolution than the Porifera.

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ИЗВОД

ХИПОТЕТИЧНА ПРВОБИТНА ЖИВОТИЊА, ПРАМЕТАЗОА: ТЕЛОМЕРАЗНА АКТИВНОСТ КОД СУЊЂЕРА (PORIFERA)

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Сунђери (Porifera) представљају најниже коло метазоа, које карактерише изразита пластичност у одређивању еволутивних линија, и најближи су таксон хипотетичној првобитној животињи, названој прометазоа, од које су дивергирале еволутивне линије метазоа. У првом приступу разјашњавању молекулских механизама који контролишу прелаз од ћелијске линије са претпостављеном неограниченом способношћу растења ка соматским ћелијама, које старе, одређена је активност теломеразе, као индикатора бесмртности. Проучавања су рађена на морским демоспонгијама *Suberites domuncula* и *Geodia cydonium*, са ткивом *in vivo*, али и *in vitro*, коришћењем приморфног система. Приморфи се граде од дисосованих ћелија које су задржале способност пролиферације. Нађено је да је активност теломеразе у ткиву оба сунђера висока. На основу овог и додатних налаза, може се претпоставити да је одвајање еволутивне линије ћелија сунђера, које старе, од линије бесмртних репродуктивних/соматских ћелија проузроковано губитком контакта са ћелијским адхезионим факторима. Укључен је први доказ који сугерише да је коначни прелаз од теломеразно-негативних ћелија које старе, ка ћелијској смрти проузрокован апоптозом.

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