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Aquatic Invertebrate Cell Culture

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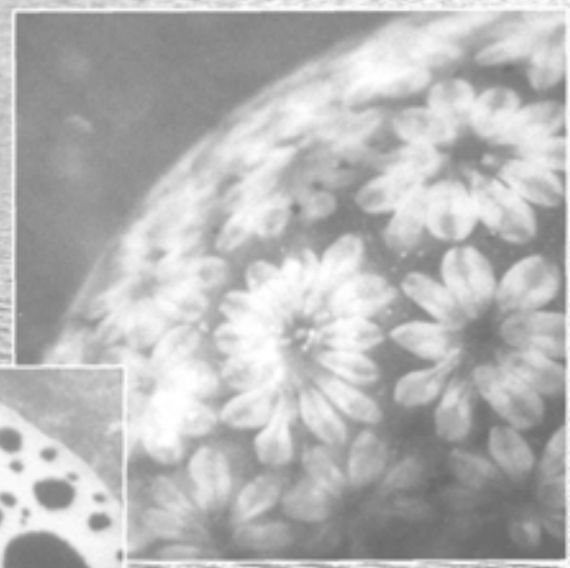

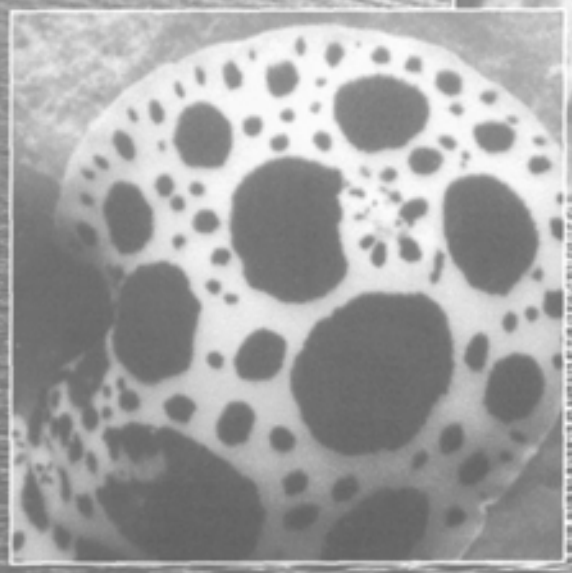

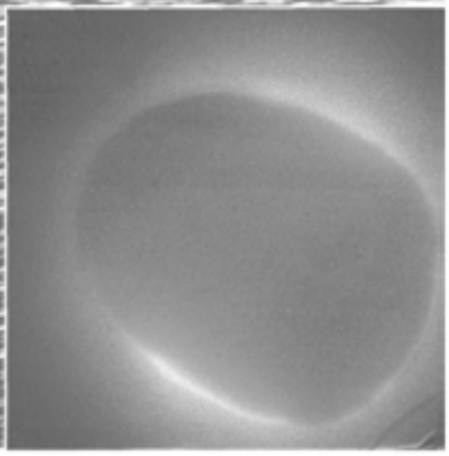

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Primary cell culture from a sponge: Primmorphs

W. E. G. Müller and M. R. Custódio

9.1 INTRODUCTION

Metazoa are multicellular, heterotrophic and diploid organisms that develop anisogamously. The diploid zygote develops by mitotic cell divisions to a ball-like larva, the blastula (Simpson, 1984). All metazoan phyla meet these criteria. Porifera (sponges) are the most ancient, extant metazoan phylum. The questions about the relationships between the different metazoan phyla in general and between the members of the lowest metazoan phylum (the Porifera) and those of higher invertebrates in particular, as well as for the ancestor(s) of Metazoa among the Protozoa, remained for a long time under debate. Some authors favoured the idea that sponges had unicellular ancestors different from those of other Metazoa (polyphyly) (Margulis and Schwartz, 1995), while other scientists (e.g. Morris, 1993) believed that multicellular animals evolved only once (monophyly). Applying molecular biological data, we could experimentally establish that all Metazoa are of monophyletic origin (Müller, 1995).

Within the Metazoa, the Porifera comprise one plesiomorphic character which is not found in any higher metazoan phyla; they have in almost all of their cells high levels of telomerase activity (Koziol *et al.*, 1998). In higher Metazoa the chromosomes lose about 50 to 200 nucleotides of telomeric sequence per cell division, as a result of the semi-conservative nature of DNA replication. The cells therefore undergo only a defined number of divisions and fall in a state that is termed 'replicative senescence' (Hayflick, 1997; Berube *et al.*, 1998) which marks the end of its proliferative capacity. However, in some cell types, especially those of germ and stem lineages as well as in most of the tumours, the telomerase enzyme is permanently active and helps to maintain telomeric length by synthesizing the telomeric sequence. In all other cells this system is active during the early stages of embryonic development, but is inactivated when the cell terminally differentiates. In sponges, the fact that cells are provided with high telomerase activity implies that they do not show a clear distinction between germ and somatic cell lineages, as found in all other

Metazoa (see the review by Müller, 1998a, b). This becomes evident by the use of 'somatic' cells (e.g. choanocytes) to build reproductive structures (Gallissian, 1988). Moreover, this could also explain the exceptional long lifespan of some sponges, which can reach more than 1,500 years (Lehnert and Reitner, 1997). Nevertheless, until now no report on neoplastic diseases in sponges exists (De-Flora *et al.*, 1995).

Despite the presence of high levels of telomerase activity, which could mean indefinite cell growth, until now only sponge-cell maintenance has been achieved from *Hymeniacidon heliophila* (Pomponi and Willoughby, 1994), *Latrunculia magni-fica* (lian *et al.*, 1996) and *Suberites domuncula* (Müller *et al.*, 1996). The reasons for the fact that sponge cells *in vitro* remain in a resting stage might be found in the experimental approach to establish a single cell culture (Pomponi and Willoughby, 1994; Ilan *et al.*, 1996) and in the culture conditions.

Based on our previous findings, which indicated that single cells from *Geodia cydonium* and *Suberites domuncula* are telomerase negative, we concluded that suspension cultures of single sponge cells are more difficult to establish than those with tissue-like aggregates. This implies that cells from sponges require stimuli from cell-cell and/or cell-matrix contact in order to proliferate. Recently, we provided experimental data on the successful establishment of a sponge cell culture from *S. domuncula*. Detailed experimental data were also given recently (Custodio *et al.*, 1998, reviewed in Müller *et al.*, 1999). In addition, data were presented which demonstrate that the proliferating cells in primmorphs can be used for production of bioactive compounds and for monitoring of environmental hazards.

9.2 ESTABLISHMENT OF PRIMMORPHS

9.2.1 Sponge

Specimens of the marine sponge *Suberites domuncula* (Porifera: Demospongiae: Hadromerida) were collected in the Northern Adriatic near Rovinj (Croatia), and then kept in aquaria in Mainz (Germany) at a temperature of 16ºC for more than 1 year (Fig. 9.1A and B). All specimens live on the hermit crab *Pagurites oculatus* (Decapoda: Paguridea) which resides in shells of the mollusc *Trunculariopsis truncu-lus* (Gastropoda: Muricidae). The outer surface of these shells is covered by shallow pits measuring 1 x 1 mm (Fig. 9.1C).

9.2.2 Media

Natural sea water was obtained from Sigma (Deisenhofen, Germany). The compositions of CMFSW and CMFSW-E were given earlier (Rottmann *et al.*, 1987). Where indicated, the sea water was supplemented with antibiotics (100 IU ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin); seawater/antibiotics.

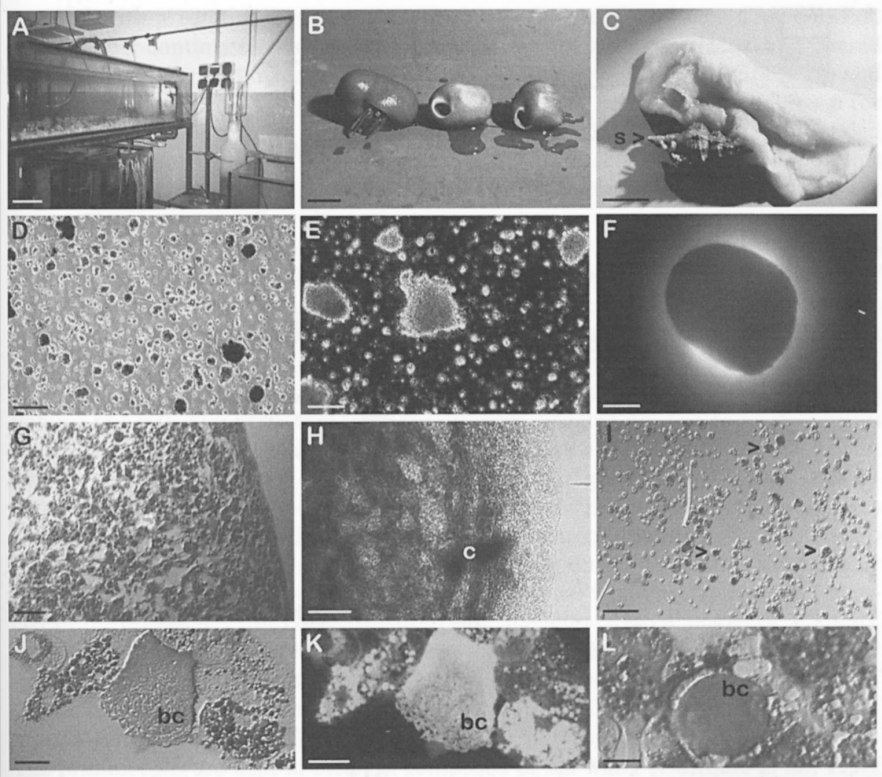

Fig. 9.1. Formation of primmorphs from cells of the sponge *Suberites domuncula*. **A.** Aquarium in which specimens of the marine sponge *S. domuncula* are kept under laboratory conditions for more than 1 year (width of bar = 20 cm). **B.** Three specimens of *S. domuncula* (bar = 1.5 cm). **C.** A specimen of *S. domuncula* from which the tissue has been removed from the shell of *Trunculariopsis trunculus* (label s); within the shell the hermit crab *Pagurites oculatus* resides (bar = 2 cm). **D.** Cell aggregates formed after transfer of dissociated cells into sea water/antibiotics (bar = 100 μm). **E.** Irregular aggregates after 1 day in sea water/antibiotics (bar = 100 μm). **F.** Primmorphs (bar = 2 μm). **G.** Cross-sections through a primmorph which are stained with Ziehl's fuchsin (bar = 50 μm). **H.** Canal-like structure (label c) in a primmorph (bar = 50 μm). **I.** Immunocytochemical detection of proliferating (BrdU-labelled) cells from primmorphs; three cells which have undergone division during the labelling period are marked (arrow heads) (bar = 100 μm). **J-L.** capsule (labels bc) filled with bacteria and surrounded by sponge cells; panels **J** and L were stained with Ziehl's fuchsin and inspected with visible light or by fluorescence microscopy (λ550-600 nm) (bars = 20 μm).

9.2.3 Dissociation of cells and formation of primmorphs

A schematic outline of the procedure is given in Fig. 9.2. All cell-culture dishes and tubes were sterilized and the media filtrated through 0.2-μm polycarbonate filters. Tissue samples of 4 - 5 cm^3 are submersed in Petri dishes in seawater and cut into 1-mm^3 cubes; these are transferred into 50-ml conical tubes (Falcon No. 2070) filled with CMFSW-E (ratio tissue to medium 1:10). After gentle shaking for 20–30 min

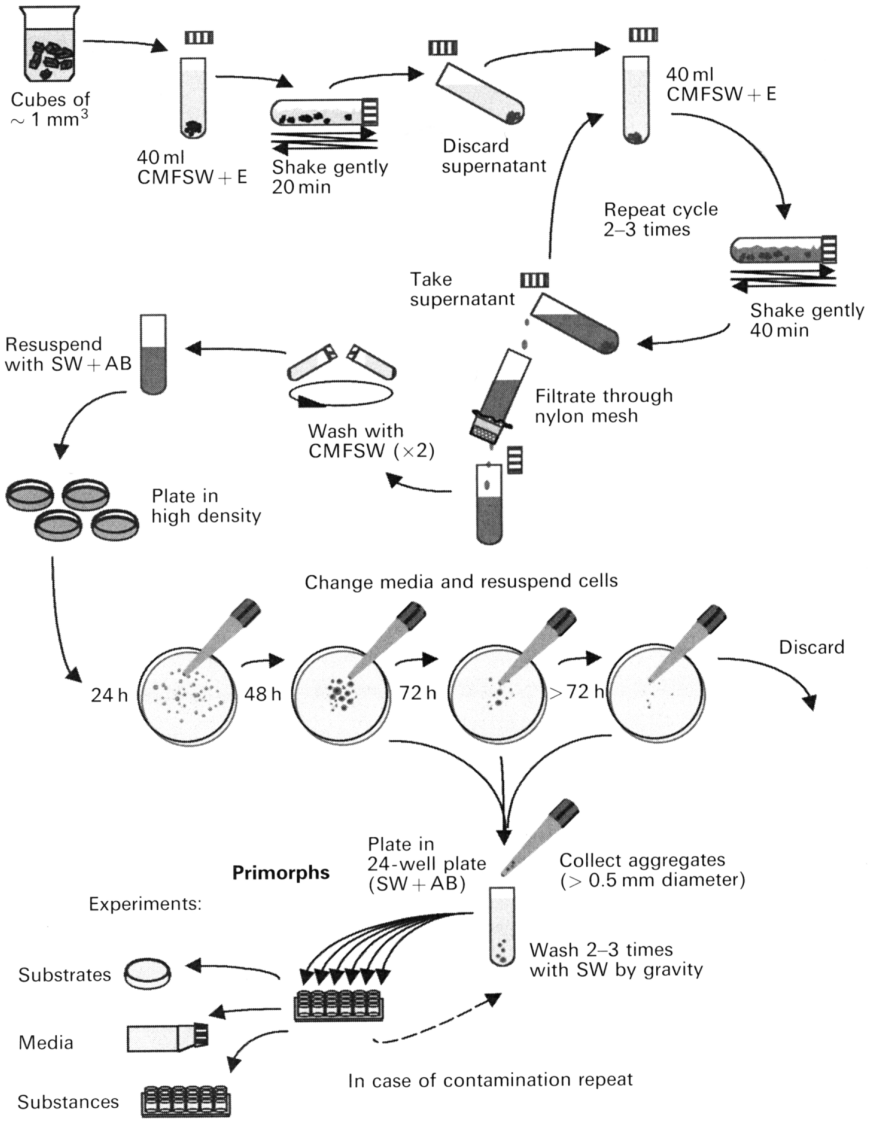

Fig. 9.2. Scheme for the generation of primmorphs from dissociated cells of the sponge *Suberites domuncula*. Single cells are obtained from tissue by dissociation in CMFSW-E. After filtration through a nylon net the cells are transferred into CMFSW and plated at high cell density in sea water supplemented with antibiotics (SW + AB). Culture medium is replaced daily by fresh sea water/antibiotics. Primary aggregates of a diameter of at least 0.5 mm is collected, washed twice in sea water using gravity alone to separate aggregates and debris. The aggregates obtained are transferred again into new Petri dishes. After formation of primmorphs, they are placed into 24-well plates with sea water/antibiotics. After 3 weeks the primmorphs can be used for experiments. Details are slightly modified according to Müller *et al.* (1999).

at 16ºC with a rotating agitator, the solution is discarded and new CMFSW-E is added. After continuous shaking for 40 min the supernatant is collected and filtered through a 40-µm mesh nylon net; this process of shaking in CMFSW-E (40 min) and filtration is repeated once. The cells are obtained by centrifugation (500 x g for 5 min) and washed once in CMFSW. The cells in the final pellets are resuspended in sea water/antibiotics to a density of 1.5-2.0 x 10^6 cells ml^{-1} and 6ml of this suspension are added to 60-mm Petri dishes (Falcon No. 3004). The cultures are kept at 16ºC.

Two-thirds of the culture medium are replaced daily by fresh sea water/antibiotics; the cultures with the cell clumps formed are gently agitated to avoid adhesion of cells to the plate. Primary aggregates of at least 0.5 mm in diameter are collected as soon as they are formed and are washed twice in 15-ml tubes (Falcon No. 2096) filled with 10 ml of sea water using gravity alone for the separation of aggregates and debris. The aggregates obtained are transferred again into new Petri dishes (total volume of 6 ml). This collection from the dishes is repeated until no more aggregates are formed or other organisms (mostly protozoans) are observed on the dishes. These aggregates are called primmorphs (see next paragraph).

After the formation of primmorphs they are placed into 24-well plates (Nunclon™, Nunc No. 143982) with 1 ml of sea water/antibiotics and one to two primmorphs per well. The medium is changed daily during the first 2 weeks, later only once or twice a week. All pipettings are performed with Pasteur pipettes (diameter of the openings: 2 mm) or plastic tips (with openings cut to diameter of 2-3 mm). After at least 3 weeks in culture, the primmorphs can be used for the experiments; for example, to test suitable substrates or culture media, or to determine the effect of certain agents on cell proliferation and DNA synthesis. One example is the potential morphogen, the endothelial-monocyte-activating polypeptide (Pahler *et al.*, 1998b). The primmorphs are kept in medium composed of sea water (Custódio *et al.*, 1998; Müller *et al.*, 1999), supplemented with 0.1 per cent (v/v) of Marine Broth 2216 (Difco).

9.2.4 Histology

Single cells and small aggregates are obtained by chemical dissociation (Fig. 9.1D). After two washing steps to remove most protozoa, which preferentially adhere to the plastic surface of the culture dishes, the cells are transferred into sea water/antibiotics. The diameter of the cell aggregates increases steadily after an incubation period of approximately 3 days (Fig. 9.1E). After a total treatment/incubation period of about 5 days, primmorphs are formed from cell aggregates. During this time, the aggregates have an irregular surface after a further incubation for 3-5 days. During the phase of primmorph formation they contract to round-shaped bodies, 1-2 mm in size, leaving behind detritus and dead cells (Fig. 9.1F). In the initial phase the primmorphs remain round-shaped; after an incubation of longer than 3-4 weeks approximately half of them adhere to the surface of the culture dish.

Cross-sections through the primmorphs revealed that they are surrounded by an almost complete layer of epithelial-like cells (Fig.9.1G).The cells which compose the

squamous epithelium of the primmorphs are pinacocytes as judged from their flattened, fusiform extensions and their prominent nucleus (Simpson, 1984); the size of the cells ranges from 15 to 20 μm. The cells inside the primmorphs are primarily spherulous cells. They have a diameter of 40-45 μm and are characterized by large round vacuoles which occupy most space of the cells. The other cells of a size of 55-60 μm 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 (Müller, 1982).

9.2.5 Subcultures of primmorphs

The primmorphs can be kept in culture by constantly renewing the medium, seawater/antibiotics. Under such conditions the primmorphs remain in a functional state by synthesizing DNA for more than 5 months.

Either immediately after formation or after 5 months, the primary primmorphs can be dissociated again using CMFSW-E. The single-cell suspension again forms aggregates and subsequently small primmorphs if they are transferred into seawater/ antibiotics; these are termed secondary primmorphs. The kinetics of secondary-primmorph formation is identical to that seen for primary primmorphs. In the absence of Ca^{2+}, using the medium CMFSW, the single cells obtained from primary primmorphs after dissociation readily attach to the surface of glass dishes. For optimal attachment to plastic, the dishes have to be scratched moderately using the tip of a pipette or a plastic rubber (not shown).

9.2.6 Long-term cultivation of primmorphs in the aquarium

Primmorphs are usually kept for at least 4 weeks in an air incubator at 16ºC. Then, they can be transferred into culture chamber slides (Nunc No. 177453) and can be cultivated further in aquaria together with other sponges (e.g. *Suberites domuncula, Geodia cydonium),*sea urchins (e.g. *Psammechinus microtuberculatus)* or sea cucumbers (e.g. *Cucumaria planci).* The animals in the aquarium (volume = 130 1) are kept in artificial sea water (Tropic Marine sea salt from Dr Binder GMBH, Wartenberg, Germany). The sponges (usually twenty *S. domuncula* specimens are kept in one aquarium) are fed with 4ml of phytoplankton (Marin Niedere Tier Futter from Amtra Aquaristik, Rodgau, Germany) twice a week; twice a month the sea water is supplemented with vitamins and trace elements (Vitamin & Mineral Supplement from Kent Marine, Marietta, GA). In addition, the hermit crab *Pagurites oculatus* (Decapoda: Paguridea) which resides in shells of the mollusc *Trunculariopsis truncu-lus* (Gastropoda: Muricidae) is present in all specimens of *S. domuncula;* they are fed with krill and animal plankton (Krill Pacifica from Petfood, F. Hundt, Wuppertal, Germany), 5 g every 3 days (Fig. 9.1A). Under such conditions, most of the primmorphs attach to the culture dishes and start to rearrange their cells into a higher organization state.

In the aquarium, the primmorphs form new monaxonal spicules (styles, surrounded by organic matrix material), which positively stains with the trichome stain Astrin (Pancer *et al.*, 1996). In addition, first formation of canals and a dermal membrane development is seen after cultivation of the primmorphs in the aquarium for 3 weeks (Fig. 9.1H). Occasionally, cell clumps are found within the primmorphs and spherulous cells are released from them. 1t is intriguing to assume that these clumps contain particles/molecules which have to be eliminated.

9.3 CHARACTERIZATION OF PRIMMORPHS

9.3.1 Level of telomerase activity in cells depending on the culture conditions

As reported earlier (Koziol *et al.*, 1998), sponge cells undergo a transition from the telomerase-positive to the telomerase-negative state after dissociation into a single-cell suspension. The level of telomerase activity has been determined in cells during formation of primmorphs from a single-cell suspension (Custodio *et al.*, 1998). Cells in natural-tissue association contain high levels of telomerase activity; a quantitative analysis revealed an activity of 8.9 TPG units 5 x 10^3 cell equivalents^{-1}. In cells which had been left for 14h in the dissociated single-cell state, the enzyme level dropped to 0.9 TPG units 5 x 10^3 cells^{-1}. However, in cells from primmorphs (used 10 days after formation from single cells) a telomerase activity of 4.7 TPG units 5 x 10’ cells ' is seen. These data confirm that cells, if removed from the tissue assembly, lose their telomerase activity. As already postulated (Koziol *et al.*, 1998; Wagner *et al.*, 1998), single cells will recover after formation of tissue-like bodies (primmorphs) and turn from the telomerase-negative to the telomerase-positive state.

9.3.2 Immunocytochemical detection of BrdU incorporation in cells of primmorphs

The BrdU-labeling-and-detection assay (Gratzner, 1982) is used to demonstrate that the cells organized into the primmorphs regain the capacity to proliferate (Custodio *et al.*, 1998). As a measure for proliferation, the cells are incubated for 12h in the presence of BrdU. Then, the incorporation of BrdU into DNA is detected by an anti-BrdU monoclonal antibody.

The BrdU-positive cells, undergoing DNA synthesis, are stained brownish in their nuclei (Fig. 9.1I); in a control assay the antibody recognizing BrdU was omitted and under this condition no staining is observed (not shown). Experiments revealed that suspensions of dissociated cells which had been kept for 1 day in CMFSW do not contain any cell that underwent DNA synthesis. The percentage of BrdU-positive cells present in cell aggregates formed from single cells after 1 day in culture is low; only 6.5 per cent are counted to be positive. In contrast, the number of DNA-synthesizing/proliferating cells present in primmorphs is high. The number of BrdU-positive cells in primary primmorphs is 33.8 per cent and in ‘older’ primmorphs, after 1 month in culture, 22.3 per cent. These data document that cells

which are reorganized into tissue-like primmorphs undergo DNA synthesis and very likely subsequently also cell division (Müller *et al.*, 1999).

9.3.3 Presence of bacteria in the primmorphs

Most sponge species, perhaps with the exception of the calcareous sponges, live in symbiosis/commensalism with bacteria, cyanobacteria and zooxanthellae (reviewed in: Simpson, 1984; Bigliardi *et al.*, 1993). In all species where bacteria have been identified, they are scattered within the mesohyl (mesenchyme) or are present intracellularly (reviewed in Simpson, 1984). In *Suberites domuncula* primmorphs the bacteria are found in clusters which are compartmented in capsules formed from unknown material (Fig. 9.1J-L). Striking is the fact that these bacteria survive the initial antibiotic treatment and the capsules remain in intimate contact with sponge cells even after dissociation (Fig. 9.1L), although they are seemingly not a part of any particular cell type ('bacteriocytes'). The capsules containing the bacteria are found both with dissociated cells from tissue and from primmorphs. A study of serial thin sections and freeze-fracture replicas of *Petrosia ficiformis* (Poiret) (Demospongiae) mesohyl revealed specialized cells (bacteriocytes) housing symbionts within large vacuoles. Adhesion sites between the symbiont envelope and vacuolar membrane are compared with similar differentiations previously described in insect endocytobiosis (Bigliardi *et al.*, 1993).

As a further support, total RNA from sponge tissue as well as from primmorphs was isolated, size-separated (Wiens *et al.*, 1998) and stained with ethidium bromide. The RNA pattern revealed four major bands of sizes of 28S, 23S, 18S and 16S, found both in the tissue and in the primmorphs. The 28S RNA represents the major rRNA species from the large subunit and 18S RNA the major species from the small subunit known from higher eukaryotic cells, while the 23S RNA and the 16S RNA correspond to the major rRNAs from bacteria (Müller *et al.*, 1999).

These two sets of data indicate that bacteria are present in tissue from the sponge *Suberites domuncula* as well as in primmorphs obtained from cells of the same species. It should be emphasized that no sign of bacterial infection is seen in the primmorphs; it appears most likely that the growth of the microbes is controlled by the sponge cells and hence supports the view that some sponges live in symbiosis with a distinct bacterial flora (Müller *et al.*, 1981; Althoff *et al.*, 1998).

9.3.4 Expression of a sponge gene as a marker for the origin of the cells

Considering (i) the fact that in the past attempts to establish cell cultures from sponges have never been tested for their true sponge origin and (ii) the risk of a sudden appearance of eukaryotic micro-organisms of the phylum Labyrinthulomycota in those cultures (lian *et al.*, 1996), a molecular marker for an unequivocal identification of the cells was applied in the *Suberites domuncula* primmorph culture. For these experiments, we selected a gene that has been shown to be relevant for environmental monitoring, a member of the cytochrome P-450 (CYP) superfamily (Gokseyr and Forlin, 1992). The CYPs belong to a superfamily of structurally and

functionally related haemoproteins, NAD(P)H-dependent mono-oxygenases, that metabolize numerous endogenous but also exogenous substrates (e.g. steroids, fatty acids, drugs, carcinogens and peptides). The prominent subfamilies are CYP1A (inducers such as aromatic hydrocarbons), CYP2B (phenobarbital), CYP3A (glucocorticoids) and CYP4A (clofibrate) (Nebert *et al.,* 1991). The specific gene from *S. domuncula* belongs to the latter subfamily. It is termed *SDCYP4* (accession number Y17816).

The size of the sponge CYP mRNA is approximately 1.9 kb, as identified by Northern Blot analysis. RNA was extracted from tissue of *Suberites domuncula* as well as from primmorphs of the same sponge species. The Northern-blot experiment using *SDC YP4* as a probe revealed that in both samples only one band is detected with a size of 1.9 kb demonstrating that the cells present in the primmorphs originate from *S. domuncula* (Müller *et al*., 1999).

9.4 APPLICATION OF PRIMMORPHS

9.4.1 Bioindicator

The approximately 5,000 currently known species belonging to the Porifera constitute the most dominant animal phylum found in the marine hard-substrate benthos. They are sessile filter feeders and are exposed to the aqueous environment in a manner not observed in any other metazoan phylum. As an example, a sponge specimen of 1 kg may filter 24,000 l per day. Therefore, it can be predicted, and it has been proven, that sponges have developed efficient strategies to resist unfavourable environmental loads (Müller and Müller, 1998).

Recently, the two sponges *Suberites domuncula* and *Geodia cydonium* were shown to be suitable bioindicator organisms to monitor dissolved heavy metals (e.g. cad-mium (Cd)) (Müller et 1998; Wagner *et al.,* 1998) or polychlorinated biphenyls (Wiens *et al.,* 1998). As biomarkers for the detection of environmental stress, the heat shock proteins HSP70 (Koziol *et al.,* 1996; Schroder *et al.,* 1999) and DnaJ (Koziol *et al.,* 1997) or putative SOS proteins (e.g. the AidB-like protein) (Krasko *et al.,* 1998) were used.

From mammalian systems it is known that Cd causes an impairment of DNA synthesis (Beyersmann and Hechtenberg, 1997). Therefore, we investigated if in the primmorph system Cd causes a reduction of DNA synthesis as well. Primmorphs (4-weeks old) were exposed to 100 ng ml^{-1} (corresponding to 163.2 ng ml^{-1} of $CdCl_2$) to 3 $pgml^{-1}$ of Cd in sea water/antibiotics for 0 - 3 days in 24-well plates. At a concentration of 1 pg ml ', a reduction by 63 per cent of the extent of DNA synthesis is seen after 2 days~if compared with the controls. In a concentration-dependent study, it was found that 0.3 pgml ' of Cd caused a reduction of DNA synthesis by 74 per cent, a value of inhibition which increases further at higher Cd concentrations during the 2-day incubation period (Müller *et al.,* 1999).

9.4.2 Production of bioactive compounds

Sponges are known to be rich sources for bioactive compounds (Sarma *et al.*, 1993). It is still under discussion if the sponge cells, the bacteria or both partners produce these compounds (Proksch, 1994). Some of them-e.g. the nucleotides arabinosyluridine (Bergmann and Feeney, 1951), arabinosylcytosine (Müller *et al.*, 1972, 1977) and arabinosyladenine (Müller *et al.*, 1975)-have been introduced into clinics. However, a wider application of those bioactive secondary metabolites from sponge, which cannot be chemically synthesized, for potential human use is hampered by the fact that the amount of defined starting material is limited. Two solutions are available. First, cultivation of intact sponge's taken from the sea in tanks-this is no problem for freshwater sponges but dificult for marine sponges, or in the field (Osinga *et al.*,1998). Second, *in vitro* cultivation of sponge cells, if possible with their unicellular symbionts/commensals, as described here. Sponges can be selected as sustainable sources for a wider use in the isolation of bioactive compounds for therapeutical purposes. In the case of toxic proteins, these difficulties do not exist. The proteins can be prepared recombinantly from all sponge species, as already demonstrated earlier for cathepsin from *Geodia cydonium* as an example (Krasko *et al.*, 1997).

Until now one bioactive compound has been isolated from *Suberites domuncula* which displays haemolytic activity, the toxic protein Suberitine (Cariello and Zanetti, 1979). By the test system described (Cariello and Zanetti, 1979), the titre of this compound was determined in the crude extract as well as in primmorphs (cultured for 5 weeks) from this sponge species. The determination revealed a haemolytic activity in the crude extract from the tissue of 4.5 TU mg^{-1} and from primmorphs of 3.5 TU mg^{-1} (Müller *et al.*, 1999). In addition, when the primmorphs are transferred to culture chamber slides and kept in aquaria, we can observe the formation of an inhibition halo, an area free of micro-organisms around the aggregate (Uriz *et al.*, 1996). Moreover, the production and liberation of spherulous cells demonstrates that these cells can retain the capability to produce and discharge possible bioactive compounds, even when not part of a fully organized sponge.

9.5 CULTIVATION OF PRIMMORPHS UNDER PRESSURE

9.5.1 Effect of ethylene

The rationale of one of our recent studies (Krasko *et al.*, 1999) was to investigate the effect of ethylene on sponge metabolism. Ethylene is known to serve as an energy source for some bacteria (e.g.*Paracoccus denitrificans*, Crossman *et al.*,1997), and to contribute to plant growth (reviewed in Abeles *et al.*, 1992). In addition, we showed that starvation of *Suberites domuncula* and also of their cells results in the induction of apoptosis (Wagner *et al*,1998). To determine the effect of ethylene on sponge cells, primmorphs from *S. domuncula*, containing proliferating cells (Müller *et al.*,

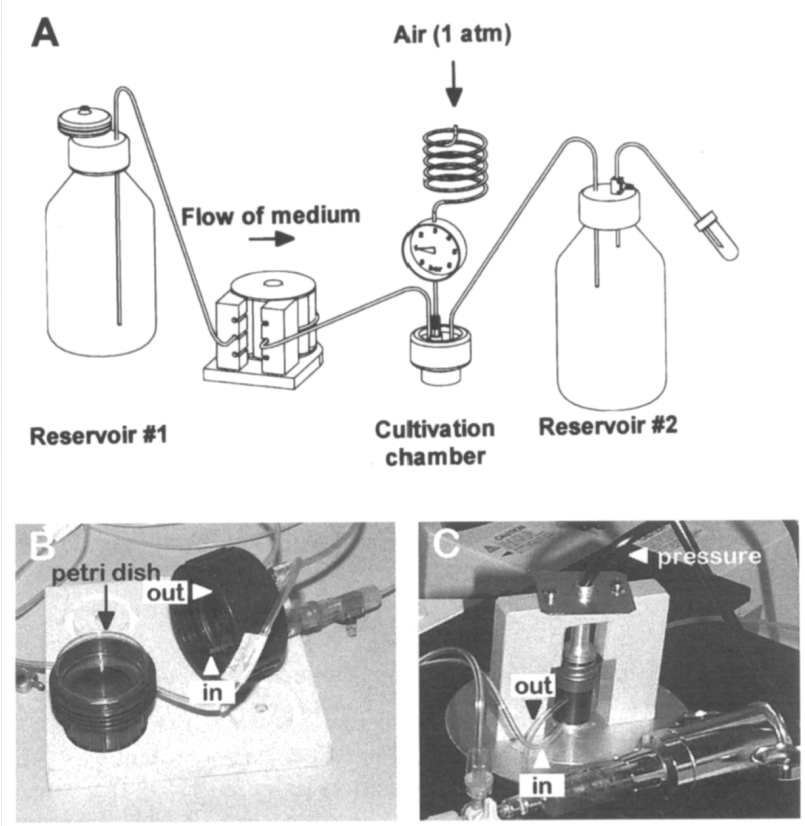

Fig. 9.3. Cultivation of primmorphs under pressure. **A.** Pressure was generated by air onto the culture chamber. Medium was pumped into the culture chamber from reservoir No. 1, filled with medium (and, if indicated, with ethylene) by a tube pump and finally collected again in reservoir No. 2. **B.** The culture chamber which contains the primmorphs has a diameter of 3.5 cm and is filled with 6 ml of medium; the tubes pumping the medium in (in) or releasing it out (out) are marked. **C.** Pressure chamber for the determination of the changes of $[Ca^{2+}]$; in response to ethylene. The arrow marks the position where the cover glass was inserted into the pressure chamber. The tubes through which the ethylene-enriched sea water was injected into (in) and extruded from (out) the chamber are indicated.

1999), were kept under pressure in the absence or presence of ethylene to imitate natural conditions.

The primmorphs were kept under pressure of 1 physical atm (Fig. 9.3). The pressure in the culture chamber was generated by air. In the studies using ethylene, reservoir No. 1 was filled with medium and 5 μM of ethylene (adjusted from a stock solution of 1 mM ethylene). The solution was pumped at a rate of 1 ml h^{-1} through the culture chamber; the extruded medium was collected in reservoir No. 2 (Fig. 9.3A). The culture chamber of a size of 3.5 x 1 cm contained the primmorphs in 6 ml of medium (Fig. 9.3B). To determine the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), cell on the cover glass were transferred into the pressure chamber and

the determination of the change of $[Ca^{2+}]$; was determined as described (Perovic *et al.*, 1999) (Fig. 9.3C).

Using this approach, we showed that ethylene reduces the extent of apoptosis caused by starvation (Krasko *et al.*, 1999). In addition, two molecular markers for the effect of ethylene were chosen. As a first molecule, the sponge gene, related to the plant stress-induced gene *HEVER*, which was found to be ethylene-responsive (Sivasubramaniam *et al.*, 1995), was selected. This sponge gene was cloned and expression studies demonstrated that it is strongly induced after exposure of cells to ethylene. The second gene selected was the Ca^{2+} calmodulin-dependent protein kinase II (CaM kinase II) which plays a central role in transduction of Ca^{2+} signals in cells. In previous studies, it had been shown that $[Ca^{2+}]$; changes rapidly in response to exposure of agonists for the metabotropic glutamate receptor (Perovic *et al.*, 1999). The CaM kinase II is activated by increase of intracellular Ca^{2+} concentration, $[Ca^{2+}]$; and also causes gene expression (Ryden and Beemon, 1989). Calmodulin has been shown to play a crucial role in integrin-mediated signal transduction in sponges (Wimmer *et al.*, 1999) via activation of CaM kinase II. Also, the expression of the CaM kinase II gene was found to be up-regulated after exposure to ethylene (Krasko *et al.*, 1999).

9.5.2 Cloning of ethylene-responsive proteins from *Suberites domuncula*

The complete cDNA, encoding the ethylene-responsive protein from *Suberites domuncula*, termed *SDERR*, is 1,106 nt long and has a potential open reading frame from the putative AUG initiation codon at nt 39-41 to the stop codon at 957-959, that encodes a 306 aa long polypeptide (Fig. 9.4A). The deduced aa sequence of the putative ethylene-responsive protein termed ERR_SUBDO has a putative size of 32,704. The bipartite nuclear targeting signature is present between aa_{63} and aa_{79}; Fig. 9.4A. Northern-blot analysis performed with the sponge *SDERR* clone as a probe yielded one prominent band of approximately 1.4 kb, confirming that a full length cDNA was isolated (Fig. 9.4B).

A databank search with the deduced aa sequence, ERR_SUBDO, revealed a high identity (similarity) to the plant sequence, the ethylene-inducible protein from *Hevea brasiliensis* (Sivasubramaniam *et al.*, 1995) of 61 per cent (82 per cent), to the 31.4-kDa protein of *Schizosaccharomyces pombe* of 66 per cent (81 per cent) and to the protein MTH666 from *Methanobacterium thermoautotrophicum* of 60 per cent (76 per cent).

9.5.3 Levels of expression of ethylene-responsive protein

In the absence of ethylene, the expression of the gene SDERR, encoding the ethylene-responsive protein, is low (Fig. 9.4B). However, after an incubation of 1 day in the presence of ethylene the expression increased significantly (1.8 fold) and reached a maximum after 3 days (6.6-fold) (Krasko *et al.* 1999). A similar pattern is seen for the expression of CaM kinase II. The steady-state level of this enzyme is low

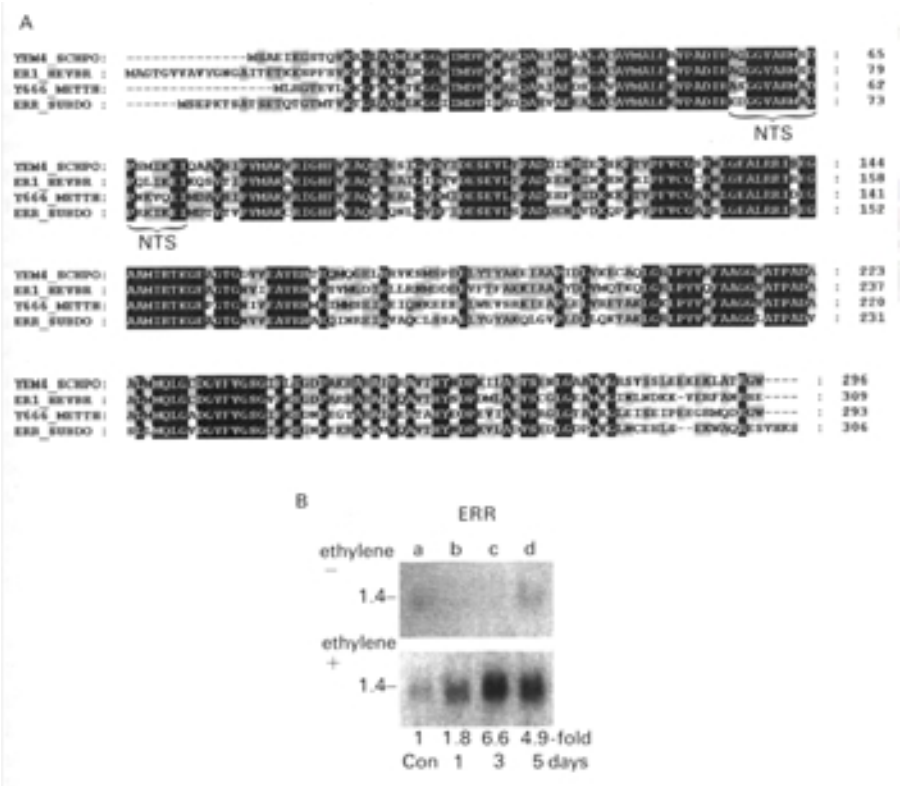

Fig. 9.4. Deduced ethylene-responsive protein from *Suberites domuncula* and its increased expression in response to ethylene. **A.** The deduced sponge sequence, ERR_SUBDO, was aligned with the following proteins: the ethylene-inducible protein from *Hevea brasiliensis* (ER1_HEVBR; accession number Q39963), the 31.4-kDa protein of *Schizosaccharomyces pombe* (YEM4_SCHPO; O14027) and the protein MTH666 from *Methanobacterium thermoautotrophicum* (Y666_METTH; O26762). The alignment was performed using the CLUSTAL W program. Residues of aa, identical among all sequences, are shown in inverted type; those present in at least three sequences are shaded. The location of the bipartite nuclear targeting signature (~ ~ ~ [NTS]) is indicated. **B.** Effect of ethylene on the expression of ethylene-responsive protein (ERR). Primmorphs were incubated for 0 day (Con; lane a), 1 day (lane b), 3 days (lane c) or 5 days (lane d) in the absence (ethylene:-) and presence of 5 μM ethylene (ethylene: +). Then Northern-blot analyses to estimate the level of expression of the genes were performed using the probe for the ethylene-responsive protein, *SDERR*. RNA was extracted and 3 pg of total RNA was size separated; after blot transfer hybridization was performed with the *SDERR* probe.

in the absence of ethylene and increase drastically after 3 (5.5-fold) to 5days (6.1-fold) (not shown).

These results show for the first time that, among Metazoa, sponges are provided with a signaling cascade in which ethylene activates cell metabolism and gene expression.

9.6 DISCUSSION OF THE SYSTEM

In a rational approach we could demonstrate that after dissociation the single sponge cells lose the telomerase activity and in consequence their intriguing property to be (to a large extent) immortal (Koziol *et al.*, 1998; Wagner *et al.*, 1998). In addition, in a series of approaches we failed to identify supplementations for the culture medium with respect to growth factors known to be required for invertebrate and lower vertebrate cell growth (data not given). Based on these facts, we postulate that, for an efficient culture of dividing sponge cells, the following prerequisites are necessary: viable cells, provision of cell-cell and/or cell-matrix contact and suitable nutrients (perhaps delivered as debris from dead homologous cells). These criteria have been met with the establishment of primmorphs. Knowing from earlier studies that sponges live in a symbiotic- and/or commensalic relationships with bacteria (Müller *et al.*, 1981; Althoff *et al.*, 1998) and/or algae (Gilbert and Allen, 1973), it was reasonable to develop a procedure by which sponge cells retain the ability to maintain this relationship to bacteria and, if present, also to algae. Therefore, the cells were dissociated and allowed to reaggregate under conditions which facilitate the preservation of their potential symbionts and allow the extrusion of debris and, very likely, also foreign pro- and/or eukaryotes. The debris was observed deposited at the rim of the free-floating primmorphs, while the foreign pro- and/or eukaryotes were found to attach to the surface of the culture dishes.

The sponge cells, assembled in primmorphs, become telomerase positive and show DNA synthesis. Hence they regain the prerequisites for cell growth, perhaps for unlimited proliferation. One cause for this transition from the telomerase-negative to the telomerase-positive state can be seen by the fact that the cells have recovered physiological contact with neighboring cells. In addition, they can attach to the homologous extracellular matrix and in consequence can arrange a functional organization.

The observation that the primmorphs comprise cells which undergo DNA synthesis even if they have been cultivated in sea water/antibiotics without any further supplements was unexpected. This observation can be explained by adopting the assumption that some cells within the primmorphs die and their resulting fragments are taken up by phagocytosis. It is well established that archeocytes, choanocytes or spherulous cells are active in phagocytosing cell debris (for a review see Simpson, 1984), a histological finding which is also supported by enzymatic data (Krasko *et al.*, 1997). Furthermore, it has been shown that in sponges spherulous cells have the ability to secrete the organic content from their vacuoles (Garrone, 1978) which supposedly can also be used as nutrients for the surviving cells. The fact that under the conditions described here no nutrients have to be added to the primmorphs in the sea-water medium does not exclude future potentially successful attempts to identify suitable growth factors for a sponge-cell culture. Studies in this direction are in progress.

Based on the data presented it is postulated that exogenous and/or endogenous factor(s) cause the transition from telomerase-positive (DNA synthesis positive) to telomerase-negative (DNA synthesis negative) cells with the consequence of an

induction of apoptosis. Recent data support this notion (Wagner *et al.,* 1998). The first apoptotic gene, MA-3, has been identified in the sponge *Geodia cydonium* (Wagner *et al.,* 1998). Based on the data available it appears likely that the process of apoptosis can be induced by the loss of binding the integrin receptor to the extracellular matrix, as demonstrated in vertebrate systems. From studies with mammalian cells it is known that the phospholipids phosphatidylserine and phosphatidylinositol are exposed on apoptotic cells (Rigotti *et al.,* 1995). These ligands bind to receptors that are SRCR (Krieger and Herz, 1994); therefore, it can be assumed that cells which expose SRCR receptors and are telomerase positive (DNA synthesis positive) phagocytose telomerase negative (DNA synthesis-negative) cells. SRCR receptors have already been identified in sponges (Pancer *et al.,* 1997; Pahler *et al.,* 1998a). Until now, no experimental data have been available on factors which might be involved in the expression of SRCR receptors. Hence, it can be suggested that both apoptotic cells and bacteria, or other organisms which act as pathogens for sponges, are eliminated via binding to scavenger (SRCR) receptors and serve as suitable nutrients for the support of the cell metabolism and as a signal for the initiation of division in cells organized in sponge primmorphs. In addition, it is evident that the sponge cells require cell-cell contact for DNA synthesis and growth. The primmorph system, described here, can be considered to be a powerful novel model system to study basic mechanisms of cell proliferation and cell death.

To conclude, the primmorph system described by Custodio *et al.* (1998) can be used in the future for a variety of applications in the following main directions: as a bioreactor to produce bioactive compounds from sponges and in environmental monitoring, as well as for the detection of potential cytostatic compounds causing a transition from telomerase-positive to telomerase-negative cells. Major tasks for the future include the establishment of continuous cell lines and cell clones from sponges and the further elucidation of the interaction between sponge cells and their symbionts/commensals at the biochemical level. In addition, proliferation factors have to be discovered and feeding of the cultures along with the development of defined culture media, needs further investigation.

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9.8 DISCLOSURE STATEMENT

A patent application has been filed with the title 'Herstellung von Primmorphen aus dissoziierten Zellen von Schwammen, Korallen und weiteren Invertebraten: Verfahren zur Kultivierung von Zellen von Schwammen und weiteren Invertebraten zur

Produktion und Detektion von bioaktiven Substanzen, zur Detektion von Umweltgiften und zur Kultivierung dieser Tiere in Aquarien und im Freiland' (AZ 198 24 384).

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