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Cell culture from sponges: pluripotency and immortality

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Sponges are a source of compounds with potential pharmaceutical applications. In this article, methods of sponge cell culture for production of these bioactive compounds are reviewed, and new approaches for overcoming the problem of metabolite supply are examined. The use of embryos is proposed as a new source of sponge material for cell culture. Stem cells are present in high amounts in embryos and are more versatile and resistant to infections than adult cells. Additionally, genetic engineering and cellular research on apoptotic mechanisms are promising new fields that might help to improve cell survival in sponge-cell lines. We propose that one topic for future research should be how to reduce apoptosis, which appears to be very high in sponge cell cultures.

Introduction

Sponges produce bioactive secondary metabolites (Figure 1) that play several ecological roles (for example, as aids in competition for space with other sponges, as deterrents against predators and as antifouling agents [1]). These metabolites have potential uses in new pharmaceuticals or cosmetics (for examples see [2]) and might have uses in a variety of other applications. The main difficulty in producing new medicines from sponges is the lack of of sufficient metabolites for both research and market needs. At present, culturing sponge explants or cells under controlled conditions is being investigated with the aims of (i) understanding the mechanisms underlying the production of metabolites of interest and (ii) supplying the pharmaceutical industry with the target compounds.

Obtaining sponge biomass by harvesting the sponges directly from the sea is not feasible in most cases because a massive extraction of these animals would harmfully impact environment. The chemical synthesis of the target metabolites on an industrial scale could be achieved but is usually not feasible because of the compounds' complex chemical structures, which make synthesis too difficult or expensive [3]. Transfection of the genes involved in the production of some bioactive substances to a cultivable microorganism, such as *Escherichia coli*, has also been explored. However, the large number of genes involved in sponges' metabolic pathways greatly complicates this process, and the current results are not conclusive [4]. If these metabolic pathways, or even parts of them, were understood, then heterologous expression of these pathways in faster-growing hosts would be the most effective route for the production of target metabolites [5].

As an alternative, several sponge culture methods have been used with variable success. These include (i) mariculture (several species of sponges have been cultured in their natural habitat based on the techniques learned from previous experiences in bath-sponge mariculture) [6], (ii) cultivation of sponge explants under controlled or semi-controlled environmental conditions (closed or semi-open systems) [7] and (iii) *in vitro* cultures, under totally controlled conditions, of explants, primmorphs or dissociated cells [8–10].

In this review, we focus on the culture of sponge cells as a direct source of bioactive substances. In these cases, a prerequisite for biotechnological production is a clearly defined system for culturing dissociated sponge tissue or cells that are free of bacteria, yeast, and protozoa contaminants. At the cell level, most sponge culture methods have been based on suspended sponge cells [11] or cell aggregates derived from cell suspensions; the latter are called primmorphs [9]. In these studies, primary cell cultures were obtained successfully, but continuously dividing cell lines could not be maintained. The main problems encountered were the difficulties in keeping the cultures axenic (free of bacteria, fungus and yeast), stimulating cells to divide continuously, reducing cell mortality and obtaining pluripotent cells. Cell-culture studies focused on the following main issues: (i) assaying different antibiotic cocktails and methods for isolating sponge cells, (ii) developing optimal growth media with added growth precursors and (iii) using archeocytes for the cultures because of their assumed pluripotency [11].

In this review, we propose new approaches that might help to solve some of the difficulties encountered in cell-culture studies and open new possibilities, such as using embryos and larvae as a new source of sponge material for culture and focusing future research on the mechanisms of sponge-cell apoptosis.

Cell culture from adult sponges

Several studies have described protocols for obtaining a primary cell culture from sponges. However, no continuous cell lines have been obtained from sponges [12], partly because of the lack of essential information about the biology and physiology of the species under culture. There is insufficient knowledge about the requirements for and

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Figure 1. Example of a Mediterranean sponge (*Dysidea avara*) that produces a bioactive compound (avarol) with anti-inflammatory activity.

the biochemical mechanisms involved in cell growth; the procedures for reducing contamination to a minimum; and the methods for obtaining viable pluripotent cells.

Primary cells should be grown, rather than harvested. The composition of the growth media, including specific growth factors or particular molecules, could improve the culture success. In several studies, a basal medium for animal cell cultures was used and supplemented with new constituents that are supposed to be crucial for the sponge cells (e.g. amino acids, vitamins, salts, glucose, organic supplements, hormones and growth factors) [11]. For instance, addition of pyruvate, vitamin C and sodium chloride has been reported to significantly increase cell viability [13,14]. Other studies modified the concentrations of the constituents to find the optimal medium empirically. Reducing glutamine and selenium concentrations in the standard medium appeared to result in greater DNA-, protein- and esterase-activity signals [15].

Furthermore, several studies demonstrated the positive effect on cell viability and growth of adding growth factors. Phytohemagglutinin, a conventional mammalian mitogen, stimulated cell division in *Hymeniacidon heliophila* (Table 1) [10]. Compared with controls, insulin, a wheat-germ agglutinin, and the *Ulex europaeus* agglutinin improved cell viability in cultures [16]. However, none of these studies succeeded in providing an ideal specific medium in which sponge cells could grow continuously.

Table 1. Taxonomical placement and reproductive features of the sponge species mentioned in the review

Species	Class	Order	Reproductive mode
Hymeniacidon heliophila	Demospongiae	Halichondrida	larviparous
Axinella corrugata	Demospongiae	Halichondrida	oviparous
Suberites domuncula	Demospongiae	Hadromerida	oviparous
Ephydatia fluviatilis	Demospongiae	Haplosclerida	larviparous
Negombata magnifica	Demospongiae	Poecilosclerida	larviparous
Dysidea avara	Demospongiae	Dictyoceratida	larviparous

A partially unsolved problem in long-term sponge cell culture is the contamination by protozoans and bacteria [12]. Such contamination shortens the culture duration and thus prevents a continuous cell line from being maintained. Removing microorganisms from the sponges is particularly difficult because of the wide presence of bacteria in sponge tissues. In many cases, bacteria are sponge symbionts, whereas in other cases they are incorporated from the environment as a source of food. Physical separation of the sponge cells from microorganisms before setting up the cell culture and the use of antibiotics during cultivation are the best methods commonly used for preventing culture contamination [11].

For a continuous cell line, cells need to be 'immortal' (continuously dividing) and pluripotent (able to differentiate into several specialized cells). Stem cells have unlimited capacity for cell-division, self-renewal and producing differentiated progeny [17]. In sponges, molecular evidence for the existence of stem cells has been provided. Two genes (noggin and the gene that encodes the mesenchymal-stem-cell-like protein (MSCP)) involved in the control of stem cell functions in higher metazoans were expressed in the sponge *Suberites domuncula* (Table 1) [18]. Moreover, in the same sponge, the expressions of two marker genes for pluripotent cells were highly expressed in oocytes and embryos (receptor tyrosine kinase (RTKvs)) and in gemmules (embryonic development protein (EED)) [18,19]. Pluripotent cell types in sponges are thesocytes in resistance gemmules and blastomers in embryos (Figure 2). In adult sponges, archeocytes have largely been considered to be pluripotent cells, but evidence for this is still missing.

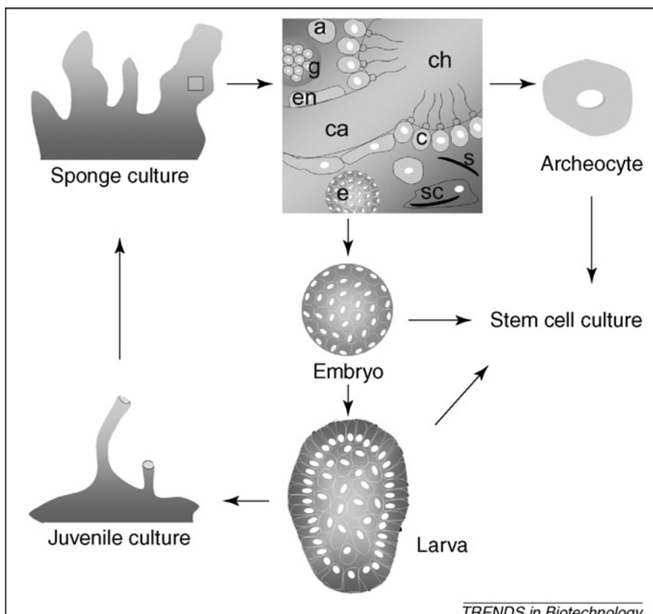

Figure 2. Schematic representation of different sponge material for stem cell cultures. Adult sponges have differentiated cells (e.g. choanocytes, pinacocytes and sclerocyetes) and pluripotent cells (archeocytes). Ripe sponges harbour high numbers of embryos, which are composed of blastomeres (stem cells) that increase in number per sponge unit during the later stages of the reproduction cycle. The embryos are released as larvae, which can be cultured as a source of (i) stem cells and (ii) sponge juveniles for obtaining sponge biomass. Abbreviations: a – archeocytes; c – choanocytes; ca – canal; ch – choanocyte chamber; e – embryo; en – endopinacocyte; g – gemmule; s – spicules; sc – sclerocyte.

Several attempts to obtain sponge cell lines from pure fractions of archeocytes have been made. De Sutter and Van der Vyver [20,21] reported a method for obtaining a pure sample of archeocytes from the freshwater sponge *Ephydatia fluviatilis* (Table 1). However, a mixture of archeocytes and several differentiated cells (e.g. choanocytes, pinacocytes, sclerocytes, etc.) has been more commonly obtained after dissociation and fractionation of cell types by density-gradient methods [10,18]. Primmorphs obtained from archeocyte-enriched cell suspensions performed better (they had more-active DNA synthesis and subsequent cell division) than those originating from non-enriched cell suspensions [13]. Nevertheless, continuous cell lines from archeocyte-enriched cell cultures were not obtained: only a few cell divisions and subsequent early cell death were observed [13]. Perhaps some of the drawbacks encountered were related to the impossibility of obtaining a pure cell fraction of adult archeocytes. Recently, a new protocol of cell fractioning for obtaining enriched archeocyte fractions has been reported [22]. Cells in these fractions seemed to show a high division capacity, proved by BrdU incorporation, PCNA expression and telomerase activity [22]. However, obtaining a pure stem cell fraction from embryonic cells might be easier because most embryonic cells are stem cells and the purification process would be straightforward.

Cell culture from sponge embryos and larvae

Embryos are present in huge numbers in ripe sponges and could be a promising source of cells for continuous cultures; this is because they are composed of fewer cell types than adult cells and most of their cells are stem cells (blastomeres). Thus, obtaining a pure cell fraction of stem cells appears to be feasible (Figure 2). The use of embryos has been reported to reduce the level of exogenous bacterial contamination in cell cultures from invertebrates [12], although proliferation of symbiotic bacteria vertically transmitted from parents to embryos [23,24] might occur in embryonic cell cultures of some sponge species. However, most symbiotic bacteria of sponges are extracellular and cannot survive out of the sponge mesohyle [25].

Sponge larvae also have relatively high quantities of stem cells. In fact, larvae are mature embryos in which some cell differentiation has occurred [26]. The few cell types that can be distinguished in a larva are transient cell types that can still differentiate into adult cell types [27]. Thus, although an embryo will probably be the optimal source of stem cells, larvae also represent a promising source.

The suitability of the embryonic sponge cells for establishing cell cultures was shown by the development of primary cultures from embryonic cells of *Negombata magnifica*; these cell cultures were maintained over a longer period of time than the cultures obtained from adult individuals [16]. Although these results are promising and show that cultures from embryonic cells are more viable, this work has not been followed up.

The two main difficulties found in the study mentioned above were discriminating between the embryonic cells (stem cells) and contaminant protozoans and obtaining a continuous supply of embryos throughout the year because of the seasonal reproduction of most species [16]. The difficulty in identifying embryonic cells has been solved with the use of molecular techniques. Molecular markers enable distinction between sponge and contaminant cells [11]. Moreover, the identification of specific cell types (e.g. pinacocytes, sclerocytes and collencytes) by genetic markers in sponges is in development [19]. The supply of embryonic cells is unresolved so far. The cryopreservation of the embryos and larvae could be a solution. It was reported that the cryopreservation of larvae did not affect the initial cell viability, although later on in the culture more bacterial contamination was observed [16]. More recently, cryopreserved cell stocks of adult sponges have been used for research purposes and have showed viability comparable to that obtained with cryopreserved mammalian cells [15,28]. We thus propose that, after a massive extraction of embryos from ripe individuals, cryopreservation can provide a continuous supply of embryonic cells for experimentation.

Although embryos seem to be the optimal source of stem cells, larvae also contain stem cells in high numbers and are more easily obtained. We have recently explored how to obtain larvae from ripe adult individuals that were kept in aquaria under controlled conditions. These ripe individuals spontaneously released hundreds of larvae [29], which settled and developed into healthy juveniles. Obtaining large amounts of larvae from ripe sponges in the laboratory might enable a year-round stock of stem cells for developing cell cultures (either as isolated or aggregate cells) and thus this approach appears to be promising.

Immortal sponge cells

The use of sponge stem cells that are grown in an optimum medium will increase the chances of establishing a continuously proliferating cell line. However, a method for ensuring continuous cell division and control of cell differentiation has to be implemented.

Animal cell lines from insects and mammals are usually transformed cells with an unlimited proliferative capacity (immortal cells). In mammalians, transformed cells can be acquired from tumourous tissues or induced artificially, either by hybridization of normal cells with tumourous cells (e.g. hybridomas), submission of the cells to mutagenic agents (e.g. carcinogenic compounds, viruses or radioactivity) or transfection of the cells with oncogenes (e.g. E1 gene or PER.C6®). Occasionally, immortal cells evolve spontaneously by the mutation of normal cells growing in rich media. Analogous to animal cell cultures, in sponges growth factors have been commonly used for stimulating cell division. However, a successful immortalization of sponge cells has never been reported.

We propose that sponge-cell-culture research should not only focus on stimulation of cell division, which has been the main aim of such research, but also on prevention of cell death. Several observations suggest that sponge cells divide and die at high rates. In natural conditions, sponges continuously change in shape and experience fission or fusion events [30,31]. The same individual is capable of simultaneously growing on one side and decreasing in size on the other side. This high dynamism and plasticity does not necessarily result in absolute growth, because sponges grow slowly, but it does result in biomass rearrangement and regeneration, which might require a high rate of mitosis and apoptosis in the same individual sponge.

Fast regeneration is a feature that is commonly observed in sponges [32]. *In situ* experiments for studying the regenerative capacity of sponges showed that perforated sponges could repair their wound at faster rates than their growth rates [30]; small holes experimentally made in the sponge tissue were healed within two days [33]. In the laboratory, this phenomenon was also observed in fragmented sponges, in which tissue regeneration started just hours after cutting [34,35]. Furthermore, when the flow conditions change, specimens of *Dysidea avara* (Table 1) can change their oscula emplacement in a few days by rearranging the exhaling canals to favour water circulation through the sponge [36]. As a whole, the sponge plasticity shown under natural and artificial perturbations (e.g. damage and hydrodynamic changes) indirectly indicate rapid cell division.

DNA replication, indicating cell division, was observed in primmorphs by BrdU labeling. However, no net growth was recorded, which suggests that cells in primmorphs were dying just as quickly as they were growing [9].

This unusual cell behaviour might be one of the reasons for sponges' success across time and space [37]. Fast cell division and death might result in an organism that is mainly composed of young cells that do not malfunction because of ageing. However, further research is needed to confirm that both cell division and cell death occur at high rates in an individual sponge.

Perspectives

Thus, to develop continuous sponge cell lines, we should not only use the strong capability of sponge cells to divide but also concentrate on reducing the high apoptosis activity of sponge cells.

Studies on the apoptotic process in sponges are in progress. Several genes involved have been identified, and the same apoptotic molecules that have been described in mammals (members of the Bcl-2 family, members of the TNF family, caspases, transcription factors and various proteins) have been identified in sponges [37–39].

Previous research on mammals showed that many intracellular proteins are involved in both apoptosis activation and inhibition, and some methods for controlling apoptosis have been developed. They consist of the addition of nutritional or chemical elements to the media and the use of genetic engineering to modify the intracellular biochemistry of the cell [40]. In sponges, a decisive first step toward obtaining transfected cells has occurred with the introduction of an immortalizing agent (human telomerase reverse transcriptase [hTERT]) into *Axinella corrugata* (Table 1) cells [41]. In this study, DNA and RNA isolation revealed the presence and expression of the vector in the sponge cell suspensions. This represents a promising research line that could lead toward a continuous sponge-cell culture.

In summary, although continuously proliferating cultures from immortalized sponge stem cells have not yet been developed, the information accumulated from sponge biology, cell requirements and genetic techniques offer good prospects for future developments in this field (Box 1).

Box 1. Endeavours and key points

- The development of a continuous sponge cell line is a challenge that still needs further pluridisciplinary research.
- A successful sponge cell line would be based on continuously dividing cells, which are able to differentiate into several specialized cells (pluripotent cells).
- Sponge embryos are proposed here as a promising source of stem cells because they are mainly formed by blastomeres, which have high proliferation capacity, and are potentially able to differentiate into all the cell types present in an adult sponge (including those producing bioactive compounds).
- The process of obtaining an enriched stem cell fraction from embryos might be easier than obtaining an archeocyte-enriched fraction from adult sponges because most embryonic cells are blastomeres and the purification process would be straightforward. Moreover, as reported for other invertebrates, the use of embryonic cells might reduce the contamination by pathogenic bacteria.
- Sponge larvae contain a relatively high number of stem cells; in fact, larvae are mature embryos in which some cell differentiation has occurred. Thus, sponge larvae can also be considered as a promising source of stem cells.
- The problem of securing a supply of embryonic cells throughout the year is unresolved so far. The cryopreservation of embryos and larvae obtained from ripe sponges in the laboratory has to be studied as a storage method.
- The high dynamism and plasticity observed in sponges suggests that sponge cells divide and die at high rates in the same individual to adapt themselves to a changing micro-environment. Thus, we propose that cell-culture research should not only focus on stimulation of cell division but also on reducing cell apoptosis.

However, once a continuous sponge cell culture is obtained, the question remains as to whether the cultured cells will continue to produce the bioactive molecules necessary for new medicines. Evidence that bioactive sponge cells in suspension still produce the secondary metabolite has been reported. For instance, stevensine was detected in the primary cell culture of *Axinella corrugata* (Table 1) [28].

Sponges have an enormous potential for the development of new medical drugs. Thus, although several bottlenecks remain, efforts to develop a technology for continuous cell cultures, such as the use of embryonic stem cells to form a cell line and research into the control of apoptosis, are worthwhile and might become successful in the interim.

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