Cell kinetics of the marine sponge *Halisarca caerulea* reveal rapid cell turnover and shedding

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SUMMARY

This study reveals the peculiar in vivo cell kinetics and cell turnover of the marine sponge *Halisarca caerulea* under steady-state conditions. The tropical coral reef sponge shows an extremely high proliferation activity, a short cell cycle duration and massive cell shedding. Cell turnover is predominantly confined to a single cell population, i.e. the choanocytes, and in this process apoptosis only plays a minor role. To our knowledge, such fast cell kinetics under steady-state conditions, with high turnover by shedding in the absence of apoptosis, has not been observed previously in any other multicellular organism. The duration of the cell cycle in vivo resembles that of unicellular organisms in culture. Morphological and histochemical studies demonstrate compartmentalization of choanocytes in the sponge tissue, which corresponds well with its remarkable cellular kinetics. Coral reef cavity sponges, like *H. caerulea*, inhabit low nutrient tropical waters, forcing these organisms to filter large volumes of water and to capture the few nutrients efficiently. Under these oligotrophic conditions, a high cell turnover may be considered as a very useful strategy, preventing permanent damage to the sponge by environmental stress. *Halisarca caerulea* maintains its body mass and keeps its food uptake system up to date by constantly renewing its filter system. We conclude that studies on cell kinetics and functional morphology provide new and essential information on the growth characteristics and the regulation of sponge growth in vivo as well as in vitro and the role of choanocytes in tissue homeostasis.

Key words: Porifera, cell kinetics, cell cycle, BrdU, shedding, apoptosis.

INTRODUCTION

Sponges and other benthic suspension and filter feeders play a profound role in the food web of marine ecosystems (Gili and Coma, 1998). These organisms filter vast volumes of water and efficiently remove organic matter from the water column. On the coral reef, sponges inhabiting coral reef framework cavities play a key role in the uptake of organic matter (De Goeij et al., 2008a). Coral cavities take up massive amounts of organic matter, in the range of the average gross primary production of an entire coral reef ecosystem, of which most (>90%) is dissolved (De Goeij and Van Duyl, 2007). Sponges dominate the cover of cavity walls (Richter and Wunsch, 1999; Scheffers et al., 2004). Organic carbon consumption rates by various encrusting sponge species are the highest observed to date (De Goeij et al., 2008a). More than 90% of their daily organic carbon intake is dissolved (De Goeij et al., 2008a), which may explain why these high rates have previously not been found in studies focusing on particle feeding by sponges. 13C-tracer studies confirmed that dissolved organic matter is assimilated by sponge cells and associated bacteria within the cavity sponge *Halisarca caerulea* (Porifera: Demospongeae) (De Goeij et al., 2008b).

For this cavity sponge a major discrepancy was found between the high amount of organic carbon assimilated by the sponge (35–40% body C day−1) and the low net increase of biomass by the sponge, which is close to zero (De Goeij et al., 2008a). So what is the fate of the assimilated organic carbon in this sponge? In other words: since the sponge is not growing, where does all this carbon go? The organic carbon may be used for reproduction of the organism or the production of secondary metabolites, but may also point to a high turnover of matter. Sponges have a high plasticity, or regeneration capacity, with up to 2900 times the normal growth rate after tissue damage (Ayling, 1983) and a high telomerase activity (Koziol et al., 1998). Both features imply the potential for relatively rapid cell proliferation.

Since sponges have an integrated multicellular organization they should have control mechanisms for growth, which is the net result of an increase in cell number (cell proliferation) and cell loss. This way, organisms are able to maintain homeostasis in the various cell populations (Koziol et al., 1998). The proliferation rate is determined by the proportion of cells actively proliferating (the growth fraction) and the duration of the cell cycle. Cell loss can occur by different mechanisms, including necrosis, programmed cell death (apoptosis) and shedding of cells. Knowledge of the cell kinetics is important for understanding the growth characteristics of a cell or cell populations in tissues or organisms (Wilson, 2007). Sponges possess most of the core elements of apoptosis, including members of the caspase family.
Sponges possess various types of cell, of which the kinetic behaviour is largely unknown. These various cell types are structurally organized in the body of the sponge. The sponge body is built around a system of chambers and canals, the aquiferous system, through which water is channelled for nutrition and respiration. The chambers (choanocyte chambers) are lined by choanocytes, which are flagellated cells containing the basic pumping and filtering elements to take up material from the surrounding water. The aquiferous system is surrounded by the mesohyl, containing the granulo-fibrillar matrix and collagen fibrils, and different cell types, such as archaeocytes and spherulous cells. Although sponges lack internal organs, they may show compartmentalization, allowing or preventing the exchange of cells from one compartment to the other. The relationship of this functional organization of the sponge with the kinetic behaviour of cells, proliferation and loss, is largely unknown.

The intention of this study was to answer the questions raised by the major discrepancy of organic carbon assimilation in relation to biomass increase found in coral reef cavity sponges. Using *H. caerulea* as a model species, this discrepancy and, subsequently, the lack of knowledge on *in vivo* growth characteristics of sponges, prompted us to investigate the process of cell turnover in this sponge.

To investigate the cell kinetics in *H. caerulea* we labelled the sponge tissue *in vivo* with BrdU and analysed the cell kinetic parameters using immunohistochemical staining of BrdU in tissue sections, based on the studies of Nowakowski and colleagues (Nowakowski et al., 1989). In order to study cell loss we determined *in vivo* apoptosis by immunohistochemical staining of sponge tissue using an antibody against active caspase-3. Furthermore, general and immunohistochemical staining methods were used to study compartmentalization in sponge tissue. A combination of studies on cell kinetics and compartmentalization within the sponge tissue should provide a better understanding of the cell turnover and cellular homeostasis of *H. caerulea*.

**MATERIALS AND METHODS**

**Sponge collection**

The thin (0.8–2.5 mm) cavity-dwelling encrusting sponge *H. caerulea* (Vacelet and Donadey, 1987) was collected by SCUBA diving on the Caribbean island of Curaçao, Netherlands Antilles (12°12’N, 68°56’W) (see De Goeij et al., 2008a; De Goeij et al., 2008b). Pieces of sponge were rinsed from overhangs and coral cavity walls at 15–25 m water depth. Attached pieces of coral rock were cleared from epibionts. In a first series of experiments the sponges were stored upside down in wire cages (20 cm × 20 cm × 15 cm; maximum of four pieces per cage) to protect them from sediment accumulation and predation. Cages were stored inside coral reef cavities at 15 m water depth. In a second series of experiments sponges were kept in aquaria (100 l) at 26°C in the dark. Water flow, reef water pumped up from 10 m water depth from the reef slope, was regulated at 31 min⁻¹. Sponges were acclimatized for at least 1 week prior to incubation experiments. The viability of the sponges using this method was studied previously (De Goeij et al., 2008a; De Goeij et al., 2008b).

**BrdU incorporation experiments**

To determine the proliferation rate of cells in *H. caerulea* sponges were placed in 1.71 incubation chambers with a magnetic stirring device at 12 m water depth (first series) or in 21 incubation chambers in aquaria (second series). Sponges were continuously labelled with 5-bromo-2'-deoxyuridine (BrdU, Sigma, St Louis, MO, USA) in a final concentration of 50 μmol l⁻¹ and experiments were performed in the dark at 26°C. Sponges (*N*=3) were fixed at *t*=0, 0.5, 1, 3, 6 and 10 h after BrdU exposure. Sponge tissue was fixed in 4% buffered paraformaldehyde (4 h at 4°C), subsequently washed in phosphate buffered saline (PBS) and stored in PBS/ethanol (1:1): at –20°C.

Fixed sponge tissue was dehydrated in a graded series of ethanol and embedded in butyl-methyl-methacrylate (BMM). Sections (3 μm) were cut on an LKB2 ultra microtome (LKB Nova; LKB, Stockholm, Sweden) and collected on microscopic glass slides (10 wells; Erie Scientific Company, Pittsburgh, PA, USA).

BMM was removed using acetone. The sections were washed in PBS and incubated in citric acid (0.2% Ph 6.0, 30 min at 90°C), washed and DNA was denatured in HCl (2 mol l⁻¹, 30 min at 37°C). Tissue sections were neutralized in sodium borate buffer (pH 8.5) and washed with PBS. Blocking was done with 1% BSA (Sigma) in PBS (30 min this step and all subsequent steps at room temperature, RT). After washing in PBS, sections were incubated with mouse anti-BrdU monoclonal antibody (MuBio Products BV, Maastricht, The Netherlands; clone IIBS, 1:50 in PBS with 1% BSA, 30 min) and washed. Primary antibody was detected with the avidin–biotin enzyme complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA). Sections were first incubated with biotinylated horse anti-mouse antibody (in PBS with 1% BSA, 30 min), washed and then incubated in avidin–biotin–peroxidase complex in PBS (30 min). Peroxidase activity was visualized with DAB (0.05% with 0.015% H₂O₂, 5–10 min). The slides were washed in tap water and mounted in glycergel (DAKO, Glostrup, Denmark). The slides were examined under a light microscope (Axiovert 200, Zeiss, Sliedrecht, The Netherlands) and photographs were taken with a Zeiss AxioCam MRc5 digital camera. Several controls tested the specificity of the BrdU labelling. Mouse BrdU-labelled colonic tissue was used as a positive control. Negative controls, without primary antibody, were performed on both mouse and sponge tissue. Additionally, BrdU immunohistochemistry was performed on sponges fixed at *t*=0 and on freshly sampled tissue.

To estimate the proportion of BrdU-positive choanocytes, three tissue (1–2 cm²) samples were taken from each sponge (edge, middle and with and without osculum). From each tissue sample four sections (>1 mm apart, containing >250 choanocyte cells per section) were counted. Per sponge >3000 (4 × 250 × 3) choanocytes were counted, and three sponges were used for each time point (six time points in total). Estimates of labelling index (LI), growth fraction (GF), duration of the cell cycle (*Tc*) and duration of S-phase (*Ts*) were calculated from the continuous labelling curve, according to an iterated least squares fit to the data, using the following specified initial conditions:

\[
f(t) = GF \times \left( t + T_3 \right) / T_C \quad \text{for} \quad t \leq T_C - T_S ,
\]

\[
f(t) = GF \quad \text{for} \quad t \geq T_C - T_S .
\]

These equations are based on a ‘one population model’ (Nowakowski et al., 1989).
In order to estimate the carbon demand of *H. caerulea* choanocyte proliferation over time (μmol C cm⁻³ h⁻¹), the number of BrdU-positive choanocytes per cm³ sponge over time was determined. We assumed that the carbon content of one choanocyte is close to the carbon content of a choanoflagellate (CC) – 10 fg C μm⁻³, with a volume of:

\[ V = \frac{W^2 \times L \times \pi}{6}, \]

where \( W \) is cell width and \( L \) is cell length (μm) (Borsheim and Bratbak, 1987). Choanocytes have no obvious homologue among metazoans, but are functionally comparable to choanoflagellates, which are closely related to sponges (Leys and Eerkes-Medrano, 2006), and share the general cell structure of choanoflagellates (Maldonado, 2004). The thickness of a living and pumping sponge (filled with water) is larger (2.5 mm) than that of a fixed sponge (0.8 mm), and values were corrected \( (F) \) accordingly to compare carbon removal rates. The number of choanocytes was counted in four randomly chosen areas \( (A; 0.0214 \text{cm} \times 0.0160 \text{cm}) \) from eight sponges. The flux was calculated according to:

\[ \text{Carbon demand} = \frac{\text{Ch} \times \text{GF}}{V/T_c}, \]

with

\[ \text{Ch} = \frac{\text{CC} \times 10^9}{12}, \]

and

\[ V = A \times \text{th} \times F, \]

where \( \text{Ch} \) is the amount of choanocytes (μmol), \( V \) is the volume (cm³) and \( \text{th} \) is the thickness of a section \( (0.3 \times 10^{-3} \text{cm}) \). The percentage of choanocytes in total tissue was established using image analysing software (Axiovision, Zeiss). The 95% confidence limits are given, unless stated otherwise.

**Caspase-3**

After removal of BMM, slides were washed in PBS, incubated in citric acid and washed. The tissue sections were incubated with purified rabbit anti-active caspase-3 (BD Biosciences, Erembodegem, Belgium; no. 557091, 0.25 μg ml⁻¹ PBS with 1% BSA, 1 h at RT). The specificity of the antibody on sponges is described by Tepsuporn and colleagues (Tepsuporn et al., 2003). The slides were washed in PBS and incubated with goat anti-rabbit–biotin IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; in PBS with 1% BSA, 30 min). After washing, the sections were incubated with avidin–biotin–peroxidase complex and peroxidase activity was visualized with DAB. The sections were rinsed in tap water and mounted with Glycergel (DAKO). Immunohistochemistry without primary antibody served as negative controls.

**Extracellular matrix**

For all staining, BMM was removed with acetone as above. Sections were stained with Alcian Blue solution (pH 2.5) for 30 min, quickly rinsed with tap water and incubated with 1% periodic acid (10 min). After rinsing with distilled water the sections were incubated with Schiff’s reagent (20 min), rinsed with tap water and counterstained with hematoxylin (5 min).

For immunohistochemical staining of basement membrane components, endogenous peroxidase was blocked by incubation in methanol containing 0.3% H₂O₂ for 20 min, followed by rinsing with Tris buffered saline, pH 7.5 (TBS). Sections were incubated in citrate buffer (pH 6.0; 30 min, 80°C) and pretreated by digesting the tissue with 0.1% pepsin in 0.01 mol⁻¹ HCl (30 min, RT). Antibodies used were: polyclonal rabbit anti type-IV collagen antibody (Eurodiagnostica, no. 2233PCO; Arnhem, The Netherlands) (Havenith et al., 1987; Cleutjens et al., 1990) and polyclonal rabbit anti-laminin antibody (Eurodiagnostica, no. 2232PLA). Sections were incubated with the primary antibodies for 1 h. After washing with TBS, the sections were incubated with biotinylated sheep anti-mouse IgG (Amersham Biosciences Benelux, Roosendaal, The Netherlands; dilution 1:1000) or biotinylated swine anti-rabbit IgG (Amersham, dilution 1:1000) (1 h). Subsequently, sections were washed and incubated with horseradish peroxidase-coupled ABC reagent (DAKO; 30 min). The sections were then washed with TBS and incubated with diaminobenzidine containing H₂O₂ (3–5 min). After washing with water the sections were counterstained with hematoxylin, dehydrated and mounted with Entellan. Immunohistochemistry without primary antibody served as negative controls.

**RESULTS**

**Cell kinetics**

Fig. 1 demonstrates the percentage of BrdU-positive choanocytes generated over time after continuous *in vivo* labelling. A linear increase of BrdU-labelled proliferating cells was observed until a maximum was reached. This maximum represents the growth fraction (GF), or the proportion of the total population of proliferating choanocytes. The duration of the linear increase represents the duration of the cell cycle \( (T_c) \). The y-intercept represents the labelling index \( (LI) \), the proportion of cells in S-phase and the x-intercept shows the duration of S-phase \( (T_s) \). The line is the least squares fit obtained to the conditions for a ‘one population model’ as described by Nowakowski and colleagues (Nowakowski et al., 1989); means ± s.d.
The results of the cell kinetic analysis as well as the incorporation of BrdU in *H. caerulea* choanocytes over time correlate significantly with a ‘one population model’ (Nowakowski et al., 1989). This strongly suggests that choanocytes are the sole proliferating cell population.

**Site of proliferation**

Incorporation of BrdU into cells of *H. caerulea* was demonstrated after in vivo exposure of sponges to BrdU and immunohistochemical analysis of sponge sections sampled from different regions of the sponge. The labelled cell population consisted primarily of choanocytes, small cells of approximately 3–6 \( \mu \text{m} \) in diameter lining the well-developed choanocyte chambers (Fig. 2). Only a few BrdU-positive cells were observed scattered in the mesohyl (Fig. 2B). This pattern of BrdU-labelled cells did not change with increasing BrdU exposure time (Fig. 2A–D). Sponges without BrdU labelling (sponges fixed at \( t=0 \) and freshly sampled tissue; Fig. 2A), and the negative control (Fig. 2E) showed no BrdU-labelled cells or background labelling. Mouse BrdU-labelled colonic tissue provided additional negative and positive controls (not shown), confirming the specificity of the BrdU labelling. Per sponge, no differences were observed between the percentage of positive choanocytes per section. Similarly, analysis at various locations within the sponge (the edge or middle, with or without osculum) showed no significant differences with respect to the percentage of positive choanocytes, which is indicative of steady-state conditions, instead of a response to injury. In addition, there was no significant difference between the measurements done in situ (at 12 m water depth) and the experiments performed in aquaria.

**Cell loss by apoptosis and shedding**

The number of active caspase-3-positive cells, as a measure of apoptosis, was investigated in *H. caerulea* tissue. Only a few positive cells were found, i.e. 0–4 cells per \( 0.34 \times 10^{-3} \text{ cm}^2 \) sponge. The negative controls did not show any caspase-3-labelled cells or background staining (not shown). Apoptotic cells were not distributed evenly throughout the sponge tissue. Positive cells were located both in the mesohyl adjacent to the choanocyte chamber, and near or inside canals leading to the outflow opening (Fig. 3). The caspase-3-positive cells did not have the characteristics of choanocytes, but strongly resembled spherulous cells and archaeocytes. Some cells in the mesohyl showed active caspase-3-positive inclusions. A few small active caspase-3-positive cellular fragments were found close to choanocyte chambers and (out of the eight sponges examined) only two caspase-3-positive choanocytes were detected.

Choanocytes were found in the lumen of the choanocyte chambers and a large amount of cellular debris was found in the lumen in canals leading to the oscula of *H. caerulea* (Fig. 4). This debris
contains predominantly choanocytes and a few spherulous cells (Fig. 4D). In sponges exposed for a few hours to BrdU, the expelled or shed material, including the shed cells, was clearly BrdU negative (Fig. 4E). In contrast, sponges that were labelled with BrdU for more than 6 h showed positive staining of the shed cells and debris (Fig. 4F). These observations demonstrate massive shedding of BrdU-labelled and hence newly formed cells, and point towards their choanocyte origin. Apparently, ‘older’ cells are expelled in the lumen of the canals leading to the outflow openings, in order to maintain tissue homeostasis and prevent clogging of the choanocyte chambers due to the massive proliferation of choanocytes.

**Structural organization of H. caerulea**

General and immunohistochemical staining methods showed compartmentalization of the choanocyte chambers. Alcian Blue staining showed proteoglycan-like extracellular components at the borders of individual cells in the mesohyl and at the transition of the basal part of the choanocytes towards the mesohyl. Generally, a sharp demarcation was visualized, strongly indicating compartmentalization (Fig. 5A).

Immunohistochemistry with type IV collagen and laminin antibodies showed the presence of a compartmentalizing layer at the transition of the basal side of the choanocyte, which separates the choanocytes from the surrounding mesohyl (Fig. 5B,C). Apparently, both collagen type IV and laminin antibodies, generated against human antigens, showed specific labelling, without any background staining. Negative controls (Fig. 5D,E), performed in parallel, did not show any labelling. These results indicate the presence of common epitopes between human collagen type IV and H. caerulea components.

**Cell turnover and carbon assimilation**

Choanocytes comprise 18±1% of the total tissue volume of H. caerulea. The carbon content of one choanocyte (with an average volume of 28±2μm³) is 2.8 pg. On average, 218±44 choanocytes were counted per 0.34±10⁻³ cm² (i.e. one image field at ×400 magnification). The carbon needed to produce new choanocytes, based on the cell proliferation kinetics for H. caerulea, was found to be 13.8±0.9μmol C cm⁻³ sponge h⁻¹. This is a rough estimate and should be considered as an order of magnitude to compare with the measured organic carbon fluxes into the sponges previously found (De Goeij et al., 2008a).

**DISCUSSION**

We have examined in vivo cell kinetics of the marine sponge H. caerulea by measuring proliferation characteristics, using continuous BrdU labelling and quantitative analysis of BrdU incorporation. In addition, apoptosis was determined using caspase-3 immunohistochemistry. The tropical coral reef sponge shows remarkable proliferation kinetics, i.e. very high proliferative activity and a short cell cycle duration. The analysis of cell kinetic parameters indicates the proliferation of a single population of cells in H. caerulea, represented by the choanocytes. Remarkably, under steady-state conditions, high levels of cell loss were found, not by apoptosis but
Sponge rapid cell turnover and shedding

through shedding of predominantly choanocytes. The high cell turnover, indicated by the fast proliferation and concomitant shedding of choanocytes, corresponds well with the observed discrepancy between the high assimilation of organic carbon and the absence of a corresponding net increase in the biomass of \( H. caerulea \).

Additionally, the estimated cost of proliferation for this sponge (13.8 \( \mu \)mol C cm\(^{-3}\) h\(^{-1}\)) is of the same order of magnitude as the high flux of organic carbon removal by \( H. caerulea \) (18.5\( \pm \)2.8 \( \mu \)mol C cm\(^{-3}\) sponge h\(^{-1}\)) previously shown (De Goeij et al., 2008a). In line with the cell kinetics, the histochemical studies demonstrated two other features of \( H. caerulea \) that correspond well with a crucial role of choanocytes in this sponge. The morphological studies identify the choanocytes as the predominant proliferating and shed cells and strongly suggest compartmentalization of choanocytes in the sponge tissue. The morphology of the sponge \( H. caerulea \) was found to correspond well with its remarkable cellular kinetics.

**Cell kinetics**

The extremely high proliferation rate in vivo, indicated by the high growth fraction (46.6%), the short duration of the cell cycle (\( T_c = 5.4\) h) and the short S-phase (\( T_S = 0.5\) h), is remarkable and has – to our knowledge – not been observed to date in sponges and other multicellular organisms.

There are only a few studies published on the cell kinetics of sponges and, more specifically, of choanocytes. The in vivo cell kinetics determined in our study do not corroborate well with cell proliferation rates measured for in vitro sponge systems, like cell cultures or primmorphs. In those studies choanocytes were not specifically measured, and primmorphs lack choanocytes altogether (Custodio et al., 1998). In contrast, the focus with respect to proliferating cells has been on archaeocytes to date. It is thought that archaeocytes represent the predominant, if not the only, proliferative cell type in sponges (Sun et al., 2007). Interestingly, when studying the choanocyte chamber formation in the freshwater sponge \( Ephydatia fluviatilis \), Tanaka and Watanabe found that choanocytes were rapidly dividing, with cell cycles of approximately 4 h (Tanaka and Watanabe, 1984). This clearly demonstrates the proliferation capacity of choanocytes. In another study, the cell cycle of choanocytes of the marine sponge \( Hymeniacidon sinapium \) was studied, which showed a much longer cell cycle of 20–40 h (Shore, 1971). Choanocytes of sponge larvae showed a cell cycle duration of 13–15 h (Efremova and Efremov, 1979). These studies are not easy to compare. They were performed ex situ, using artificial seawater, and it is well known that ideal conditions for keeping sponges are still not established. Moreover, very small fragments of sponge were used for the experiments, resembling in vitro more...
than *in vivo* conditions. In addition, (larvae of) freshwater sponges may be considered as quite different from (adult) marine sponges. Sponges occur in a wide variety of habitats with their own specific biotic and abiotic conditions, which may all affect their growth characteristics. The generality of the cell kinetics of *H. caerulea* needs to be confirmed by studying the *in vivo* cell kinetics of sponge species from different habitats and ecosystems.

The proliferation rate of *H. caerulea* choanocytes is extremely fast compared with that of other metazoans. *Hydra* is a well-studied lower metazoan closely related to the Porifera and is expected to have a high proliferation rate of cells because of its similar regenerative power and high turnover of cells. However, the fastest cell cycles measured in *Hydra* are 16–27 h for interstitial (stem) cells (Campbell and David, 1974), compared with 4–5 h for sponge choanocytes. The fastest proliferative tissues are found in the food uptake systems of metazoans. However, most cell cycle durations found in uptake systems are much longer than those found in the filter cells of *H. caerulea*. For example, the gill cells of a suspension feeder like the mussel have a $T_c$ of 24–30 h (Martínez-Expósito et al., 1994). The intestinal epithelium of the mouse is one of the fastest proliferative tissues found to date. But again, the cell cycle duration of 12–15 h is approximately 3 times longer than that of choanocytes (Rowinsky et al., 1977). The proliferation of the sponge choanocytes resembles that of unicellular organisms in culture like, for example, choanoflagellates. The doubling time of the choanoflagellate *Monosiga* in culture during log phase was approximately 6 h (King et al., 2003), almost identical to the $T_c$ of *H. caerulea* choanocytes. It should be noted that under natural conditions marine bacteria have doubling times ranging from 8 h to a few days (Fuhrman and Azam, 1982).

**Cellular homeostasis under steady-state conditions: cell loss by shedding of choanocytes**

The proliferation characteristics of the choanocyte cell population correspond to a steady-state situation and not to a regenerative process. No differences in proliferative patterns were found between the sponges kept in their natural environment and the sponges kept in aquaria. Tissue damage, evoking regeneration of tissue, was most likely to occur at the sponge edges (where sponges were cut), but clearly no difference in the growth characteristics was observed between sections taken from different locations in the sponge, including the edges. Additionally, the regenerative power of sponges in response to tissue damage has been shown to depend on the migration and proliferation of archaeocytes, which are considered to be the toti/multipotent ‘stem cells’ (Simpson, 1984). Since the mass and overall structure of sponge tissue do not change significantly over time, the observed cell kinetics strongly suggest a rapid cell turnover in the choanocyte compartment. In order to maintain cellular homeostasis within the sponge, an increase in cell number has to be balanced by loss of cells from the same compartment, by apoptosis, autophagy (Xie and Klionsky, 2007) or shedding.

Specific loss of choanocytes through apoptosis is very unlikely, since very few active caspase-3-positive cells were found and these were predominantly residing in the mesohyl and not in the proliferation compartment. The caspase-3-positive cells showed the morphological characteristics of spherulous cells and archaeocytes. In many of these cells, caspase-3-positive staining was observed in distinct entities in the cytoplasm. In all these cells an intact nucleus was present. Apparently, the labelled cells are not apoptotic themselves, but contain phagocytosed apoptotic bodies. Caspase-3-positive cells are transported out of the sponge by being expelled into the canals leading to the outflow openings. It is likely that these cells are the spherulous cells passing through the pinacocyte layers of canals in *H. caerulea*, as observed by Vacelet and Donadey (Vacelet and Donadey, 1987). Apoptosis may serve as part of the sponges’ waste control, by picking up apoptotic cells or cellular debris and transporting it out of the sponge, rather than playing a role in tissue homeostasis.

Therefore, most choanocytes are lost by shedding of cells from the choanocyte chambers into the central canals leading to the
osculum, or outflow opening. Evidence for cell shedding in *H. caerulea* is strongly supported by the presence of large amounts of cellular debris and choanocytes in these compartments. The presence of BrdU-labelled cells and debris after relatively long (6 and 10 h) exposure of the sponge to BrdU (Fig. 2), and the absence of BrdU staining in the material shed after relatively short (0.5–3 h) labelling indicates that this cellular material originates from the rapidly proliferating choanocytes. To our knowledge, this is the first observation of the process of cell shedding in sponges. Cell loss by shedding is well established in epithelia of the mammalian gastrointestinal tract. In human colonic tissue, for example, the proliferating cells are found in the lower two-thirds of the crypt, migrating upwards and replacing the cells in the upper part of the crypt. At the top of the crypts the excess cells lose their mutual binding, are detached from the basement membrane and are shed into the lumen (Van de Wetering et al., 2002). In *Hydra*, which are phylogenetically closer to the sponges, shedding is also observed. Moreover, similar to *H. caerulea*, shedding of tissue occurs in *Hydra* in order to maintain constant size and cellular composition of the organism under steady-state growth (David and Campbell, 1972).

*Halisarca caerulea* excretes large amounts of detritus daily (J.M.DeG., personal observation) as has been reported for several other sponge species (Reiswig, 1974; Witte et al., 1997; Yahel et al., 2003). In all of those studies, the excreted detritus was termed ‘unidentified’ feeding waste. The majority of this ‘unidentified’ waste can in fact be attributed to a high cell turnover to maintain tissue homeostasis in these sponges.

**Compartmentalization in sponge tissue**

The *in vivo* cell kinetic analysis of *H. caerulea* demonstrates that the massive proliferation is not a random phenomenon, but is restricted to a single population of cells. Cell kinetic processes require a structural and functional basis; thus in other words, the question is whether these are reflected in the morphological characteristics of the sponge. The kinetic and morphological data strongly suggest compartmentalization of choanocytes in *H. caerulea*. In the mesohyl only a few scattered proliferating cells were observed, suggesting that no exchange, migration or differentiation of BrdU-labelled cells occurs between the choanocyte compartment and the mesohyl. *Halisarca caerulea* has a distinct distribution of extracellular matrix components in differently organized compartments, as already shown in a TEM study (Vacelot and Donadey, 1987). The cells in the choanocyte chambers are delineated by extracellular matrix components that stain with Alcian Blue, like proteoglycan-like or hyaluronic acid-containing structures (Zierer and Mourao, 2000). Furthermore, the compartmentalization of choanocytes is strengthened by an extracellular matrix structure surrounding the choanocyte chambers, which seems to contain components sharing epitope homology to type IV collagen and laminin. Although no hard evidence is yet available for the identification of these components as type IV collagen and laminin, the results show a specific demarcation without background staining and a negative control without labelling. The combination of the structural organization together with the cell kinetics of a distinct cell population indicates a functional compartmentalization in the sponge. This compartmentalization might function in a variety of cellular processes, such as homeostasis, metabolism, differentiation and polarity of these sponges, in particular of the choanocytes. Further studies should be performed to investigate the presence and identification of basement membrane-like structures.

### Implications

*Halisarca caerulea* is an encrusting coral reef sponge, inhabiting coral overhangs and framework cavities, playing a key role in the recycling of nutrients within the reef framework (De Goeij et al., 2008a). Its habitat provides shelter and protection, and therefore the competitive struggle for space and food for these benthic organisms is high (Buss and Jackson, 1979). The tropical waters surrounding coral reefs are very low in nutrients and are considered to be the marine equivalent of a desert. Marine benthic organisms have adapted to their environment by becoming very efficient and opportunistic suspension feeders. Sponges are known for pumping vast volumes of water over time (Reiswig, 1974) and removing enormous amounts of matter from their environment (Gili and Coma, 1998). As a consequence, the sponge, as a benthic filter feeder, is continuously in contact with large volumes of water containing nutrients, but also potential mutagenic, toxic, viral, bacterial and physical stress factors. A relevant survival strategy to cope with this function and the hostile environment may be a high turnover of cells. Before possible structural damage of the adsorptive system occurs, the ‘old’ cells are shed and replenished by new cells. Not surprisingly, sponges have a high telomerase activity (Müller and Müller, 2003), preventing highly proliferative cells from senescence, but cells that lose their contact with other cells, for example through shedding, subsequently lose their telomerase activity (Müller and Müller, 2003). We are aware of the fact that this study was performed on only one species, but we found similar discrepancies in carbon removal rates and net growth in two other species of cavity sponges (De Goeij et al., 2008a).

Within an environment of high competition for space and of low concentrations of nutrients, the coral reef sponge *H. caerulea* maintains its body mass and keeps its food uptake system up to date by constantly renewing its filter system. This cell kinetics study on sponges provides new and essential information on the growth characteristics and the regulation of growth of sponges. Future studies should take into account the pivotal role of choanocytes in the tissue homeostasis of sponges, and their peculiar cell kinetic behaviour.

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