Chapter 13
Effect of Bacterial Infection on Stem Cell Pattern in Porifera

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Abstract Multicellular organisms derived from one common ancestor, the Urmetazoa. The only living fossils, which can testify about the earliest evolutionary processes in Metazoa on the molecular level are sponges (phylum: Porifera). The present study outlines that stem cells may play essential roles in cellular specialization, embryogenesis and sponge Bauplan formation, using the demosponge *Suberites domuncula* as a model. Data indicate that the archaeocytes represent, besides the germ/embryonic cells, totipotent stem cells. First marker genes have been identified, which are expressed in totipotent stem cells and in cells from gemmules. Furthermore, genes have been described that are characteristic for the three main cell lineages in sponges; they all originate from archaeocytes and are involved in the differentiation of skeletal cells, epithelial cells and contractile cells. Finally it is shown that after exposure to the endotoxin LPS (lipopolysaccharide) a differential gene expression occurs, leading to an upregulation of the gene encoding perforin and a concomitant down-regulation of noggin, a stem cell marker. In parallel with this process an increased phosphorylation of the mitogen-activating protein kinase p38 occurs. This modification of the p38 kinase has been quantified with a novel ELISA assay. Our data suggest that in response to bacterial infection the number of stem cells in sponges decreases.

Keywords Sponges · Cell culture · Infection · Stem cells · Primmorph · Strees-response genes · Gene expression · Sponge genes.

13.1 Introduction

Based on molecular biological and cell biological data it is now established that the approximately 30 phyla [including also the Porifera (sponges)], integrated in

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B. Rinkevich, V. Matranga (eds.), *Stem Cells in Marine Organisms*,
DOI 10.1007/978-90-481-2767-2_13,

the multicellular animal kingdom of Metazoa, share one common ancestor, the Urmetazoa (Müller et al. 1997; Müller 2001). Other than metazoan organisms, colonies of animal cells, such as choanoflagellates, display no division of labor. In Metazoa the cells have been differentiated to perform a series of functions, like digestion, sensation, contraction or secretion (Grunz 2004). The determination of metazoan cells to a distinct fate occurs via an alteration in the pattern of gene activity/expression, which thus allows a specification into distinct roles and functions. Specialized cells are the basis for pattern formation, a process during which a spatial and temporal pattern of cell activities is organized within the well-ordered organism (Nüsslein-Volhard and Wieschaus 1980). Metazoa are grouped into (i) the morphologically more diverse bilaterians, which themselves are further subdivided into Protostomia, including Ecdysozoa and Lophotrochozoa, and Deuterostomia and (ii) the non-bilaterian metazoans (phyla Porifera, Placozoa, Coelenterata and Ctenophora; Brusca and Brusca 1990).

During ontogeny a body plan is established, which also defines the main axes of the multicellular animal. From Porifera to Arthropoda (Protostomia) and Vertebrata (Deuterostomia) the level of complexity of the axes (antero-posterior ends [oral-aboral polarity], dorsal-ventral sides) increases (Müller 2005). The two phyla Porifera and Coelenterata possess only two epithelial layers, the ecto-epithelium surrounding the body and the endo-epithelium (Bergquist 1978; Garrone 1978), which encloses the digestive cavity(ies); they are termed diploblastic animals. The two groups are defined by only one apical-basal polarity (Wiens et al. 2003). The triploblastic, bilaterian animals contain in addition to these external layers a third, middle, mesodermal cell layer which originates usually from the endoderm. Body pattern formation can be studied during embryogenesis or during differentiation of embryonic cells, e.g. in three dimensional cell cultures. These morphogenetic processes are based on and controlled by differential spatial and temporal expression of genes that initiate or maintain a large number of signaling pathways (Galliot and Miller 2000).

Segmentation (a process during which very similar functional units along a body axis are formed almost simultaneously [in insects]) or somite formation (sequential formation of units along a body axis [in vertebrates]) are features of triploblastic organisms. The formation of a body axis is controlled by characteristic sets of genes; e.g. in insects the parasegments are delimited by the function of the pair-rule genes and the subsequent segments by the segment polarity genes (Wolpert 1998). Ancestors of these genes exist in diploblasts, Coelenterata and Porifera, as single molecules (Hoshiyama et al. 1998; Wiens et al. 2003).

During the past few years it was elaborated that the Porifera (sponges), as the phylogenetically oldest metazoan phylum, possess already the basic structural and functional elements required for the construction of a body plan (Perovic et al. 2003). They evolved approximately 800 million years ago (Müller and Schäcke 1996). Sponges are grouped according to the inorganic composition of their skeleton (spicules) into the classes of Demospongiae and Hexactinellida, which possess hydrated, amorphous, noncrystalline silica spicules, and the class of Calcarea, whose skeletal spicules are formed from calcium carbonate (Simpson 1984).

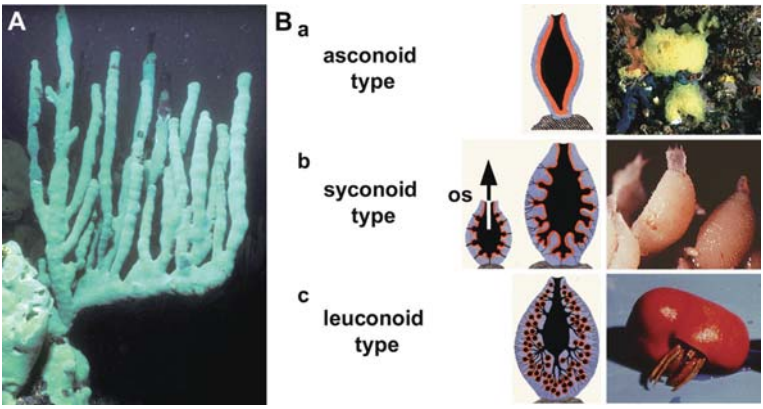

Fig. 13.1 Body plan organization in the phylum Porifera. (**A**) Sponges, here *Lubomirskia baicalensis*, conduct water from the external milieu through their inhalant openings at the surface to the exhalent apertures, the oscules. This highly complex aquiferous system is formed from differentiated somatic cells, which interact in a tuned manner through cell-cell/cell-matrix interaction. The water flow through a specimen can be visualized with fluorescein; the pulsatory extrusion of the water current can be seen (not shown). (**B**) The three different organization/body plan patterns of sponges are shown. The choanoderm layer is in *red* and the pinacoderm layer in *blue*. (a) In the asconoid type, the sack-like organization shows an unfolded choanoderm. (b) In the syconoid type the folding of the choanoderm layer results in the formation of choanocyte chambers, which are connected with the incurrent canals. After passing the chambers the excurrent canals lead the water current to the oscule (os). (c) The organization of the leuconoid type. This complex canal network is composed of discrete choanocyte chambers, connected with a network of incurrent and excurrent canals. In all three types of body organization a basal site (attachment to the substratum) and an oscular/apical pole (oscule; opening at which the excurrent canals extrude water from the central cavity) exists, which determines the axis (*arrow*). Representative species of the different organization levels are shown: (a) *Clathrina coriacea*, a calcareous sponge of the asconoid type. Magnification ×0.2. (b) A syconoid type of organization as seen in *Sycon raphanus* (Calcarea); ×3. (c) *Suberites domuncula* a siliceous sponge (Demospongiae) of the leuconoid type; ×0.5

In sponges the water flow is directed from the lateral surface of the specimens, through the porocytes and the canals to the lacunae and the choanoderm [choanocyte chambers]; Fig. 13.1. These chambers are composed of two kinds of epithelial cell layers, formed by choanocytes [flagellated cells] and by cone cells [cells with a double-conical shape, which hang into the chamber formed by choanocytes]. These layers surround the mesohyl; in this central body the sponge cells are loosely embedded in a matrix, composed primarily of collagen, galectin and glycoconjugates (Müller et al. 1997). This matrix surrounds motile archaeocytes, which are pluripotent, and other, differentiated cells, e.g. collencytes and lophocytes, that are involved in the formation of collagen as well as sclerocytes that form spicules. In addition, myocytes exist, which allow contraction of the sponge body upon mechanical irritation (Simpson 1984).

According to the folding pattern of the two epithelial layers of the pinacoderm, in correlation to the choanoderm, three types of poriferan body plan complexity

are distinguished; the asconoid, the syconoid and the leuconoid type (Bergquist 1978). The folding of the choanoderm allows the formation of spherical chambers, the choanocyte chambers, of different structural degrees (Fig. 13.1). In the asconoid type, the lateral opening(s) direct the water current to the continuous layer of choanocytes which face a single atrium and then to the oscule (Fig. 13.1B-a). This organization is seen in some Calcarea, e.g. *Clathrina coriacea*. In the syconoid type the folding pattern forms choanocyte chambers and the water flow enters the sponge through the porocytes into choanocyte chambers through which the water is pressed into the atrium and finally via the oscule again to the external milieu (Fig. 13.1B-b). This type of organization is seen in Calcarea and Demospongiae; as an example the calcareous sponge *Sycon raphanus* is shown here. The leuconoid type derives from the syconoid type of organization. Here, the porocyte openings lead from the vestibule to the connecting incurrent canals and into the choanocyte chambers. Subsequently, the water current is pressed into excurrent canals which open into the atrium and then leaves the organism via the oscule (Fig. 13.1B-c). Most sponge species, like the marine (siliceous) demosponge *Suberites domuncula* (Fig. 13.1B-c) show the leuconoid type of organization. The Hexactinellida have an organization pattern which is reminiscent of the syconoid type and are composed of a choanodermal syncytium (see: Müller et al. 2004). Recently, the biochemical and molecular basis of the axis formation in sponges, here shown with the freshwater sponge *Lubomirskia baicalensis* as example, had been demonstrated (Fig. 13.1A; Wiens et al. 2006).

By molecular biological techniques – in the meantime more than 30,000 ESTs have been identified from the sponge *S. domuncula* – and subsequent functional classification it could be disclosed that the basic strategies of body plan formation and gene expression patterns found in Porifera are characteristic for Metazoa in general (Müller et al. 2001). Furthermore, an *in vitro* 3D-cell [three-dimensional] culture system, termed primmorphs, was established (Custodio et al. 1998; Müller et al. 1999a). Culturing primmorphs is a newly developed technique to grow sponge cells *in vitro*; these cells have the potency to proliferate and to differentiate. This system allows an understanding of the roles of the morphogenetic and pattern forming genes. Amazingly the cell-cell- and cell-matrix adhesion molecules found in sponges share high sequence and functional similarity to those of higher metazoan phyla (Müller et al. 2004; Fig. 13.2). The extracellular binding sites to the ligands, but also the intracellular domains of these cell membrane receptors remained conserved throughout the animal kingdom. As it is also functionally proven, the receptors are provided with the properties of outside-in signaling (Wimmer et al. 1999b). This system allowed to study the effects of solute morphogenic factors (e.g. myotrophin; Schröder et al. 2000), or secreted molecules (e.g. epidermal growth factor; Perović-Ottstadt et al. 2004a), as well as of their receptors, that are involved in axis formation (Frizzled receptor; Adell et al. 2003b), and of transcription factors that are required for polarity formation (e.g. the organizer-specific factor *LIM homeobox protein*, Wiens et al. 2003; or Forkhead, Adell et al. 2003a) were discovered (Müller et al. 2004; Fig. 13.2).

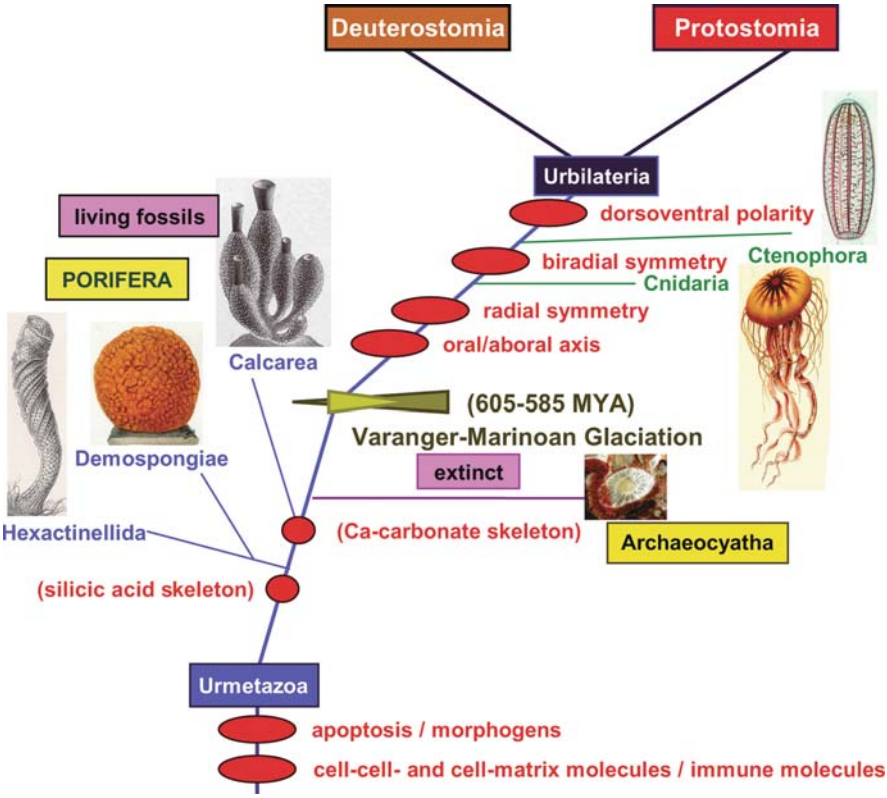

Fig. 13.2 Phylogenetic position of the Porifera within the metazoan kingdom. The three poriferan classes (Hexactinellida and Demospongiae emerged first, and later the Calcarea) evolved from the common ancestor of all metazoan phyla, the Urmetazoa. The major evolutionary novelties which have to be attributed to the Urmetazoa are those molecules which mediate apoptosis, control morphogenesis, the immune molecules and cell adhesion molecules. The three classes of Porifera are the model systems which comprise a genetic reservoir of molecules that direct including pattern formation characteristic for Metazoa; e.g. the transcription factors, paired box homeodomain molecules, LIM-class homeodomain, T-box [Brachyury] or winged helix [Forkhead]. As a sister group to the Calcarea, the Cnidaria evolved. Subsequently, the Ctenophora emerged which comprises not only an oral/aboral polarity but also a biradial symmetry. In the Cnidaria the *paired box* transcription factors have been identified. Finally the Urbilateria developed from the Diploblasts (two epithelial layers), which are built from three germ layers (Triploblasts). They diverged into the Protostomia, with the crown species *D. melanogaster* and *C. elegans*, and the Deuterostomia with *H. sapiens*. Within triploblastic animals the homeobox genes are arranged in clusters

13.2 Basis of Metazoan Pattern Formation: The Stem Cell System in Sponges

Multicellular animals are characterized by the existence of a series of differentiated somatic cells, in addition to the totipotent germ cells (Cory and Adams 1998).

During metazoan evolution the number of distinct cell types increased steadily (Müller et al. 2003a; Müller 2006). The different somatic cell types derive from the zygote through the respective stem cell stage; the differentiation proceeds in niches that form a microenvironment in which defined expression patterns of signaling molecules and local environmental factors direct the fate of the cells (Jones 2001). These niches modify their regulatory property in response to a changing environment to ensure that stem cell activities meet the needs of an organism for a given differentiated cell type. Two major types of stem cells derive from the zygote, the germline stem cells and the somatic stem cells. While the germline stem cells retain their total differentiation capacity, this properties is restricted in the somatic stem cells which gradually lose their stem cell propensity. However, recent studies indicate that this traditional view of an irreversible loss of the stem cell ability during maturation of somatic cells might not completely reflect the physiological situation (Wagner and Weissman 2004). It appears that such fixed "points of no return" do not always exist but that at all levels of differentiation from the pluripotent progenitor cells to the committed progenitors to the lineage progenitors and finally to the terminally differentiated cells, the propensity to act as stem cell is retained, even though with a decreasing intensity. In consequence, the differentiation lineages of somatic cells are dynamic and plastic and the strong distinction between embryonic stem cells and adult stem cells should be reconsidered.

It is generally agreed that the archaeocytes are the toti-/pluripotent cells in sponges from which the other cells originate; evidence was presented indicating a localization of archaeocytes not only within the mesohyl but also in the endopinacoderm layer (Borojevic 1966, 1970, 1971; Simpson 1984). Archaeocytes give rise to the major classes of differentiated somatic cells, (i) the epithelial cells, pinacocytes and choanocytes, (ii) the cells forming the skeleton, collencytes and sclerocytes, and (iii) the contractile cells, myocytes (Fig. 13.3). However, archaeocytes give not only rise to the different somatic cells but also to the germ cells from which the embryos originate (Diaz 1977 and 1979). Another line of differentiation of the archaeocytes is to the thesocytes, the totipotent cells of gemmules which are asexual propagative dissemination bodies. Pinacocytes, collencytes/sclerocytes and myocytes are cells with a low stem cell propensity, implying that these somatic cells are "terminally" differentiated (Simpson 1984).

At present, the study of embryonic stem cells in sponges is limited, since no technique to induce mass production of embryos under controlled conditions has yet been successful. As a substitution, the three-dimensional cell culture has been established for *S. domuncula* (Fig.13.4A); Custodio et al. 1998; Müller et al. 1999a. Under suitable conditions dissociated, single cells (Fig. 13.4D) form special types of cell aggregates, the primmorphs (Fig. 13.4E). They contain cells of high proliferation and differentiation capacity. Furthermore, *S. domuncula* has the capacity to propagate sexually [a free-floating egg is shown in Fig. 13.4B] and also asexually, via reproduction bodies termed gemmules (Fig. 13.4C).

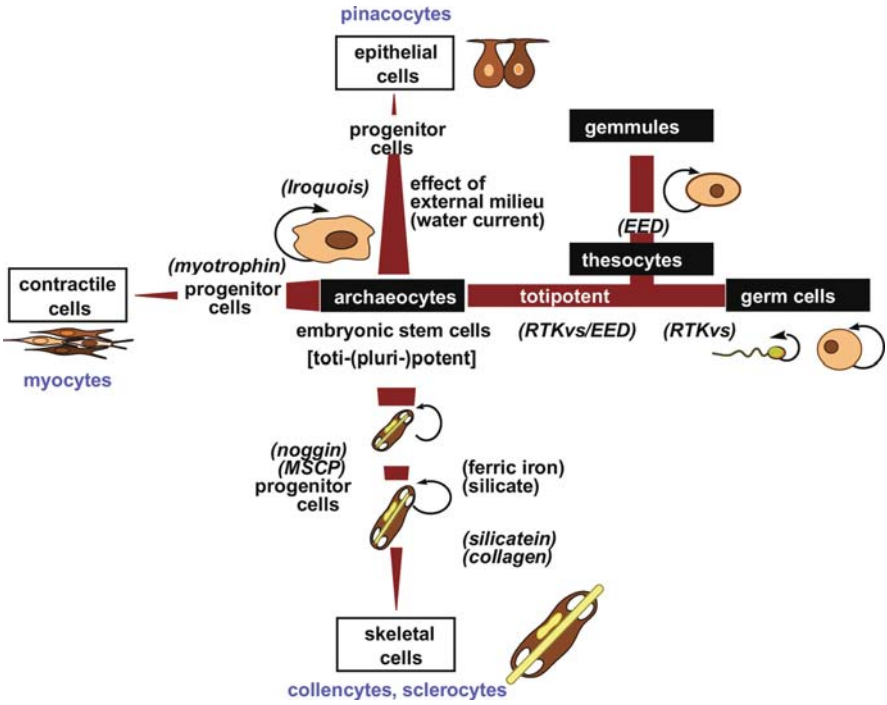

Fig. 13.3 Sponge stem cell system. Schematic outline of the development of the toti-/pluripotent sponge embryonic stem cells, the archaeocytes, to the germ cells on one side and to the three major differentiated cell types, the epithelial-, the contractile- and the skeletal cells on the other side. It is indicated that during these transitions progenitor cells characteristic for these lineages have to be passed. The (potential) factors, e.g. noggin and the mesenchymal stem cell-like protein (MSCP) on the path to the skeletal cells, which trigger the differentiation are shown. In addition it is outlined that committed progenitor cells are formed which respond to the silicate/Fe(+++) stimulus through differentiation to skeletal cells, the sclerocytes (=skeletal cells). Without losing the high level of stem cell propensity the archaeocytes change to germ cells and also to thesocytes, the dominant cells in gemmules (a sexual propagation bodies). The triangles schematically indicate the decrease in stem cell propensity during differentiation

13.2.1 Marker Genes

In order to underline the view that the metazoan stem cell concept can also be applied to Porifera, characteristic marker genes have been cloned from *S. domuncula*. The first cDNA identified whose deduced protein shares sequence similarity to mammalian stem cell markers was the mesenchymal stem cell-like protein (MSCP). *MSCP* is present in mesenchymal human stem cells; experimental evidence exists that *MSCP* is expressed in osteogenic mesenchymal stem cells (Müller et al. 2003a). The functional studies revealed that in sponges the expression of this gene is under positive control of the morphogenetic inorganic elements silicon and ferric iron (Krasko et al. 2002; Müller et al. 2003b). In addition, two

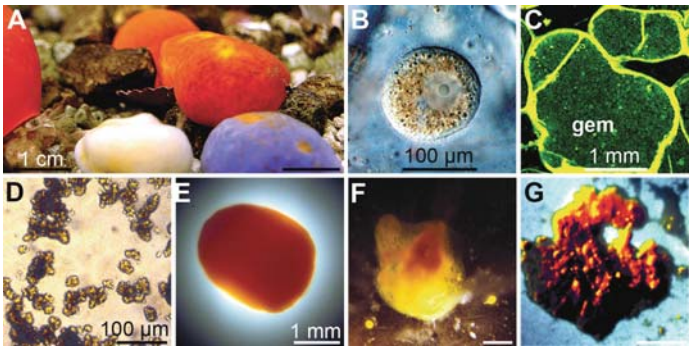

Fig. 13.4 The demosponge *S. domuncula*. It can be cultivated for over two years in aquaria (**A**). *S. domuncula* can propagate sexually (**B**- a free floating egg) and also asexually, via gemmules (**C**- a cross-section though gemmules [gem] which developed on a shell on which the sponge lives). (**D**) Dissociated single cells from this species can form aggregates which differentiate to primmorphs (**E**) after an incubation period of more than 5 days. (**F**) A primmorph which developed on galectin-coated culture dishes; it shows canal-like structures. (**G**) A gemmule which had been treated with LPS, as described under "Materials and Methods". *Size bars* for **A–E** are given; **F,G**: 1 mm

further potential genes, involved in the differentiation of stem cells in sponges, were isolated; noggin and the glia maturation factor (Schröder et al. 2003). Noggin is a glycoprotein that binds bone morphogenetic proteins selectively and antagonizes their effects. It was initially isolated from *Xenopus* and found to be expressed in the Spemann's organizer. In vertebrate development noggin is involved in the formation of dorsal mesoderm derivatives, e.g. the skeletal muscles (Smith and Harland 1992; Smith et al. 1993; Valenzuela et al. 1995; Ogawa et al. 2002; Müller et al. 2003b).

In the initial phase of formation, the *in vitro* 3D-cell primmorph system contains predominantly the toti/pluri-potent archaeocytes which can be stimulated to differentiate into four main tissue-specific directions (Müller 2006). If they are induced by the inorganic factors silicate or ferric iron, the archaeocytes give rise to the skeletal cells through an increased expression of genes encoding the structural proteins silicatein and collagen; a process which is mediated by noggin and *MSCP* (Fig. 13.3); Müller et al. 2004. Second, if archaeocytes are exposed to a morphogen (myotrophin) they are directed towards the contractile cell lineage (Krasko et al. 2002; Müller 2006). A third lineage, which gives rise to the aquiferous canal system, is induced by a physical factor; there *Iroquois* gene expression is induced as a result of an increased water current which is paralleled with the formation of canal-like pores in the primmorphs (Perovic et al. 2003). In higher metazoans the expression of the *Iroquois* genes is thought to confer identity to a particular region, and hence it can be classified to the homeotic selector genes (see: Perovic et al. 2003). The early functions of *Iroquois* in insects include the definition of the eye and notum territories, and in vertebrates the formation of the neural ectoderm. Late functions of *Iroquois* are subdividing the territories; the dorsal-lateral subdivision in

Drosophila notum and the patterning of notum bristles and wing veins, and in vertebrates the subdivision of neural tube and heart patterning. The sponge *S. domuncula* not only contains in its genome an *Iroquois* gene, which comprises not only remarkable sequence similarity to those sequences described from triploblastic animals, but also is expressed during the formation of one major morphogenetic remodeling process, the construction of the aquiferous canal system (Fig. 13.3).

Two further genes have been cloned which are of relevance for the presented work here, the thymidine kinase and the perforin-like protein. In vertebrate cells, salvage pathway phosphorylation of thymidine is catalyzed by two thymidine kinases, the cell-cycle regulated cytoplasmic thymidine kinase-1 and the constitutively expressed mitochondrial thymidine kinase-2 (Segura-Pena et al. 2007). In cellular metabolism, thymidine kinase-1 and thymidine kinase-2 serve to maintain sufficient dTTP for DNA replication and repair. Hence the level of thymidine kinase-1 transcripts reflects the extent of cell proliferation of a given cell population. We have cloned from *S. domuncula* the cytosolic thymidine kinase-1 (Fig. 13.5), since it is a well known cell-cycle-regulated enzyme of importance in nucleotide

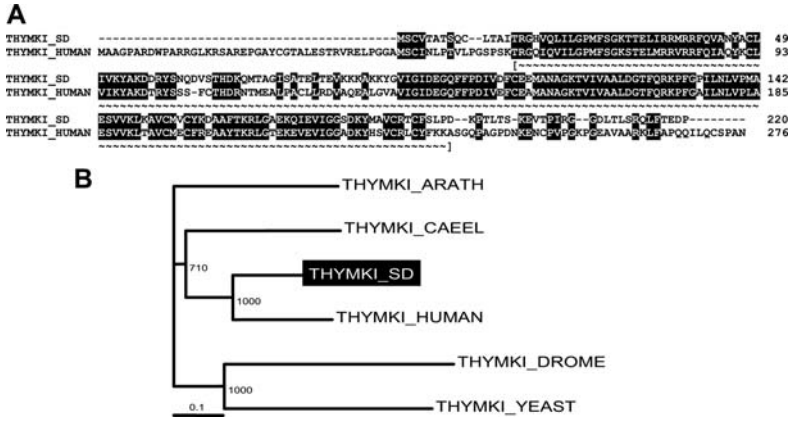

Fig. 13.5 Cytoplasmic thymidine kinase from *S. domuncula*. (**A**) The deduced sponge protein was aligned with the human soluble thymidine kinase 1 (THYMKI_HUMAN; accession number NP_003249.2; Segura-Pena et al. 2007). Amino acids, identical or similar among two sequences, are in inverted type. The borders of the thymidine kinase (EC 2.7.1.21) [tdk] domain (hamap:MF_00124; motif scan database [http://myhits.isb-sib.ch/cgi-bin/motif_scan] are marked [~~~~~~]). (**B**) These two sequences were compared with the *C. elegans* thymidine kinase family member (thk-1) (THYMKI_CAEEL; NP_501886.1), the *D. melanogaster* the putative protein CG40300-PC (THYMKI_DROME; NP_001036627.1), the *S. cerevisiae* suppressor of var1 protein (THYMKI_YEAST; 151942764), and the putative thymidine kinase from *A. thaliana* (THYMKI_ARATH; NP_568426.1). After alignment the phylogenetic tree was built, using the plant sequence as an outgroup; the neighbor-joining method (Saitou and Nei 1987) was used. The degree of support for internal branches was further assessed by bootstrapping (Felsenstein 1993). The numbers at the nodes are an indication of the level of confidence for the branches as determined by bootstrap analysis [1000 bootstrap replicates]

metabolism. The thymidine kinases-1 have a tetrameric structure in which each subunit contains an alpha/beta-domain that is similar to ATPase domains of members of the RecA structural family and a domain containing a structural zinc. The *S. domuncula* cDNA, encoding the thymidine kinase-1, was identified and was cloned as described (Perovic et al. 2003). The deduced protein with a chain length of 220 amino acids, giving a predicted size of 24,251 Da, shares highest sequence similarity to the human soluble thymidine kinase 1 (NP_003249.2); Segura-Pena et al. 2007 (Fig. 13.5A). The two sequences share 45% identical amino acids and 56% similar amino acids. This high score is also reflected by the constructed rooted phylogenetic tree; the human and the sponge gene fall into one branch (Fig. 13.5B). Separated from this branch are the (distantly) related sequences from *Drosophila melanogaster* (3% identical – 7% similar amino acids) and *Saccharomyces cerevisiae* (31–44%). Again separated from these two branches is the thymidine kinase family member from *Caenorhabditis elegans*.

Since sponges are filter feeders that are exposed to large amounts of bacteria present in their surrounding aqueous milieu, they had to develop strategies to resist and defend themselves against attacking microorganisms, among them also the Gram-positive bacteria. In a previous study we could demonstrate that in primmorphs from *S. domuncula*, the expression of a gene, termed perforin-like protein [MARKER GENE FOR DEFENSE], is activated in response to exposure towards a Gram-negative α-proteobacterium (Thakur et al. 2003). The perforin-like protein contains one EGF-like domain cysteine pattern signature and a C2 domain [thought to be involved in Ca^{2+}-dependent phospholipid binding (Davletov and Suedhof 1993).

13.2.2 Expression Pattern of Archaeocytes (Stem Cells): "Reproductive" Cells

Stem cells are self-renewing populations of cells that undergo symmetric and/or asymmetric divisions either to self-renew or to differentiate into different kinds of differentiated progeny. This minimal definition does not allow a clear distinction of stem cells from other dividing and differentiating cells (Cai et al. 2004). Surely, stem cells are provided with a high capacity for cell-cycle, for cellular protective and DNA repair mechanisms and for apoptosis. Recently genetic expression markers have been identified, which can be applied for the identification of "embryonic" cells and tissue in sponges (Müller 2006).

As outlined above, sponges have developed two propagation systems, sexual reproduction (Fig. 13.4B) and asexual reproduction by propagation bodies [gemmules] (Fig. 13.4C). The first study, using molecular markers to determine the restriction of gene expression during embryogenesis in a sponge appeared recently (Perović-Ottstadt et al. 2004b). It was found that in oocytes, morulae and blastulae/larvae from *S. domuncula* distinct genes are expressed, among them a sponge-specific receptor tyrosine kinase (RTKvs). In addition, the sex-determining protein FEM1 and the sperm associated antigen-related protein are highly expressed; in

adult animals the levels of expression of these genes are very low (Perović-Ottstadt et al. 2004b).

The asexual reproduction pattern in sponges, i.e. gemmule formation, was already early in focus of developmental biologists (Laurent 1842). Gemmule formation in sponges is induced by environmental factors, e.g. temperature, or dryness (Wagner et al. 1998). The cells in the gemmules are the thesocytes, which form a homogenous population (Fig. 13.3); it has been proposed that these cells also derive from the totipotent archaeocytes (see: Simpson 1984).

The recently identified MARKER GENES FOR TOTIPOTENT CELLS, which are highly expressed either in oocytes or in cells of gemmules, are the receptor tyrosine kinase *RTKvs* (oocytes and early larvae) and the embryonic development protein *EED* (gemmules); Müller 2006. The expression patterns of these genes are applied as tools to distinguish between differentiation levels of the cells. In tissue of adult *S. domuncula* those genes are expressed only in a few cells that are scattered in the pinacoderm. Sequence analyses of the two sponge RTKs show that the extracellular part of the *S. domuncula* kinase RTKvs_SUBDO has no obvious similarity to any other (deduced) protein hitherto submitted to the databases. Therefore, this part of the protein can currently be considered to be truly sponge-specific (Perović-Ottstadt et al. 2004b). The intracellular TK domain, however, possesses the 12 characteristic subdomains. The embryonic development protein EED of *S. domuncula* is a member of the Polycomb-group (PcG) family (Sewalt et al. 1998). *In situ* hybridization demonstrated that RTKvs_SUBDO is highly expressed in eggs and early stages of embryos in *S. domuncula* (Müller 2006). In view of these data, we suggest that the cells expressing these two marker genes represent archaeocytes, which are in the "functional" state in either fertilized eggs or cells constituting early embryos (as is the case for RTKvs_SUBDO), or form the gemmules (EED2_SUBDO); Fig. 13.6.

13.2.3 Expression Pattern of Archaeocytes (Stem Cells): Sclerocyte Lineage [Skeletal Cells]

Sclerocytes are the cells which produce the siliceous spicules, the skeletal elements of sponges. As outlined the mesenchymal stem cell-like protein (MSCP-l) and noggin can be considered as MARKER GENES FOR MULTIPOTENT STEM CELLS (Müller et al. 2003a). During differentiation to sclerocytes the genes *silicatein* and *collagen* undergo strong expression (Krasko et al. 2002).

13.2.4 Expression Pattern of Archaeocytes (Stem Cells): Pinacocyte Lineage [Epithelial Layer]

As reviewed, the pinacocyte surface layer can be looked upon as an epithelium (Müller et al. 2004). One MARKER GENE FOR THE DIFFERENTIATION

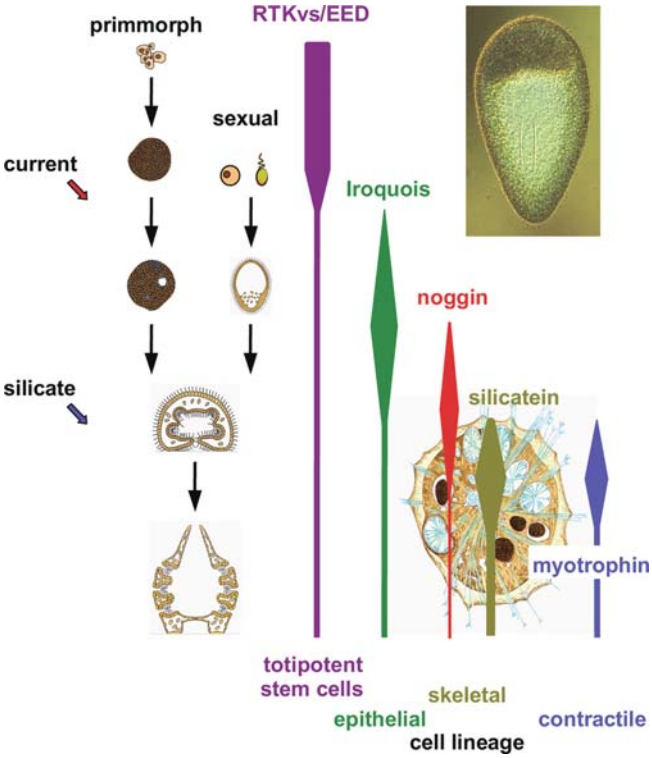

Fig. 13.6 Sequential expression of (putative) stem cell marker genes in *S. domuncula*. In primmorphs as well as in germ cells a high expression of two genes can be identified, the sponge-specific receptor tyrosine kinase (RTKvs) and the embryonic development protein (EED). They might be considered as markers for totipotent stem cells. At exposure of primmorphs to water current, the transcription factor Iroquois is expressed; this process is seen primarily in epithelial cells. Noggin as well as silicatein gene expression are provoked after addition of silicate/Fe(+++) to the culture medium; the expression is prominent in the skeletal (spicule)-forming cell lineage. In contractile cells (myocytes), myotrophin is expressed. Bars are underlaid with a photograph of a larva of the freshwater sponge *L. baicalensis* and a cross section through an entire sponge (*Craniella schmidtii*), showing embryos within the parent (Sollas et al. 1888)

of the archaeocyte stem cells to the pinacocytes has been isolated from *S. domuncula* (Perovic et al. 2003). This gene, *Iroquois* (MARKER GENE FOR THE PINACOCYTE LINEAGE) codes for a putative homeobox gene. The putative *Iroquois* transcription factor was found to be expressed in cells which are adjacent to the canal system; its expression is upregulated in primmorphs which are cultivated in strong water current (Perovic et al. 2003); Fig. 13.3. The finding that also in sponges the expression of the *Iroquois* gene is restricted to a specific tissue region, the epithelial layer of the aquiferous system, adds a further piece to the understanding of the complexity of tissue organization in sponges.

13.2.5 Expression Pattern of Archaeocytes (Stem Cells): Myocyte Lineage

Myocytes in sponges are functionally characterized as cells which synthesize the organic skeletal elements, e.g. collagen. During the progress of archaeocytes to myocytes, myotrophin is expressed in *S. domuncula* (Schröder et al. 2000). Myotrophin was first found in mammalian systems; in cardiac myocytes myotrophin stimulates protein biosynthesis (Sen et al. 1990), suggesting a crucial role in the formation of cardiac hypertrophy (reviewed in: Sil et al. 1998; Schröder et al. 2000). The sponge myotrophin shares the highest sequence similarity with the human molecule. Recombinant sponge myotrophin was found to stimulate protein synthesis by 5-fold (Schröder et al. 2000). Since myotrophin is neither expressed during embryogenesis nor in gemmules, it might be characterized as a MARKER GENE FOR THE MYOCYTE LINEAGE. After incubation of single cells with myotrophin the primmorphs show an unusual elongated, oval appearance. Furthermore, in the presence of myotrophin sponge cells upregulate collagen gene expression. We assume that the sponge myotrophin causes in homologous cells the same/similar effect as the cardiac myotrophin in mammalian cells, where it is also involved in initiation of cardial ventricular hypertrophy.

13.3 Bacterial Infection

Sponge-bacteria interactions are probably among the oldest host-bacteria interactions known, dating back more than 500 million years in time (Wilkinson et al. 1984). Several studies have revealed that permanent associations exist between certain host sponges and specific microorganisms; however their interactions remained largely unknown (Althoff et al. 1998; Friedrich et al. 1999; Schmidt et al. 2000). Moreover, sponges may also succumb to microbial and fungal infections which result in the disintegration of the sponge fibers/tissue and ultimately lead to sponge death (Vacelet et al. 1994). The fact that sponges are susceptible to microbial infection suggests that they should also be provided with mechanisms to prevent these types of diseases.

It is known that sponges possess molecules resembling those of the mammalian immune system (Müller et al. 1999b). As examples of the innate immune system, scavenger receptor cysteine rich domains and macrophage derived cytokine-like molecules have been identified which are upregulated during auto- and allografting experiments. In addition, the (2′–5′) oligoadenylate synthetase system exists in sponges (Schröder et al. 2008). Precursors of the adaptive immune system have also been identified and were shown to be functional in sponges (Müller et al. 2004). Accordingly, the expression of a lymphocyte-derived cytokine from mammals is up-regulated during non-self grafting experiments in *S. domuncula*. In the sponge *Geodia cydonium*, two immunoglobulin like receptors have been

identified which are also up-regulated during grafting experiments. These findings demonstrate that sponges contain elements for innate immune recognition (Müller et al. 1999b).

The lipopolysaccharide (LPS)-mediated pathway is an additional mechanism involved in the mammalian immune response. LPS, an endotoxin derived from the outer cell wall of Gram-negative bacteria binds to the cell surface molecule CD14 (Ulevitch and Tobias 1994). This interaction is mediated by LPS-binding protein(s) (Scott et al. 2000). CD14, a plasma membrane linked molecule presents LPS to a specific transducer resulting in an enhanced production of reactive oxygen metabolites and gene expression (reviewed in: Jiang et al. 2000). Serine-threonine directed MAP (mitogen-activated protein) kinases are essential components of the LPS-mediated pathway. These proteins can be grouped into three main families, the extracellular signal-regulated kinases (ERKs), the p38 kinases and the c-jun N-terminal kinases (JNKs) (Seger and Krebs 1995). In deuterostomes, the latter two kinases are phosphorylated after exposure of cells to LPS (Yang et al. 2000). In view of the recent findings by Müller et al. (1999c) it is hypothesized that a similar LPS-mediated immune response pathway can also be found in sponges.

In a previous study we demonstrated (Böhm et al. 2001), that cells from *S. domuncula*, that had been exposed to LPS, respond with the activation [phosphorylation] of the stress-activated MAP kinases p38 and JNK. Furthermore first insights into the types of microorganisms that are permanently associated with *S. domuncula* were gathered. In addition, we obtained evidence that in sponges LPS interacts with Gram-negative bacteria via a cell-surface bound receptor, the LPS-binding protein (Wiens et al. 2005). The cDNA was isolated and the protein expressed. During binding to LPS the protein dimerizes. Co-immunoprecipitation analysis revealed that this protein interacts with MyD88, after exposure of the animals towards LPS. The sponge MyD88 is composed of two protein interaction domains, a TIR domain (present in Toll-like receptors and in adapter molecules such as MyD88) and a death domain (present in MyD88 and the interleukin-1 receptor-associated kinase 4 IRAK). Northern blot experiments and *in situ* hybridization studies showed that the level of the LPS-binding protein does not change after LPS treatment, while MyD88 expression is strongly upregulated. As an executing molecule of this pathway (LPS-binding protein – MyD88) the macrophage expressed protein, a perforin-like molecule (Mr 74171), was identified. The cDNA was isolated; the gene is highly expressed after LPS treatment, especially at the surfaces of the animals. The recombinant protein comprises biological activity and eliminates Gram-negative bacterium (*E. coli* and BL21 [sponge-associated Gram-negative bacteria] were used); it is inactive against the Gram-positive bacterium *Staphylococcus aureus*. These data indicate that *S. domuncula* is provided with an innate immune system against Gram-negative bacteria; the ligand LPS (a pathogen-associated molecular pattern) is recognized by the pattern-recognition receptor (LPS-binding protein) which interacts with MyD88. A signal transduction is established which results in an elevated expression of both MyD88 and the macrophage expressed protein, as executing proteins.

13.3.1 Activation of p38 Kinase

The phosphorylation of the two kinases (p38 and JNK) strongly increases after exposure of the sponge tissue to LPS, a response which is characteristic of the mammalian p38 and JNK activation in response to LPS. The activation occurs rapidly within the first hour after LPS exposure. Moreover, the inhibitory effect of LPS on cells from *S. domuncula* has also been determined by measuring the incorporation of tritium labeled phenylalanine into protein fraction. At a concentration of 3 μg/ml of LPS, the reduction in the incorporation rate is already significant (Böhm et al. 2001). These findings show that the defense pathways are highly conserved between sponges and humans (Böhm et al. 2002).

13.3.2 ELISA Assay (Method)

13.3.2.1 Preparation of Sponge Total Protein Extract

Primmorphs are exposed to LPS 10 μg/ml (L2880, Sigma, Deisenhofen, Germany) and then processed as described (Böhm et al. 2000). Tissue is extracted with lysis-buffer (1× TBS [Tris-buffered saline], pH 7.5, 1 mM EDTA [ethylene diamine tetra-acetic acid], 1% Nonidet-P40, 10 mM NaF, protease inhibitor cocktail [1 tablet/10 ml] and 1 mM sodium orthovanadate) after homogenization in a mortar; a clear supernatant is obtained by centrifugation (13,000 × g; 10 min; 4°C) and then subjected to ELISA analysis.

13.3.2.2 Determination of the Phosphorylation Level of p38 by Enzyme-Linked Immunosorbent Assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) for detection of the phosphorylation level of p38 was developed. This ELISA assay is based on the ability of the anti-phosphorylated 38 (pp38) antibody to bind with high affinity to a peptid containing the TGY recognition motif (Böhm et al. 2000). The total protein extract is covalently bound to a 96-wells polystyrene plate. The complexes formed between proteins bound to the plate and the antibodies against pp38 are detected using a peroxidase-conjugated secondary antibody and the 3,3,5,5-tetramethylbenzidine (TMB) as substrate.

In detail: Flat-bottomed polystyrene 96-well Pro-Bind plates (Becton Dickinson, France) were covered with 50 μl of total protein extract in final concentrations of 5–60 μg/well during incubation for 3 h at room temperature. After washing three times with phosphate buffered saline (PBS), containing 0.05% Tween-20 (PBS/T buffer), the plates were blocked with 3% bovine serum albumin (BSA) in PBS (150 μl/well), overnight at 4°C. The level of pp38 in the protein mixture bound to the plate was detected with a polyclonal antibody against phosphorylated p38 (pp38)

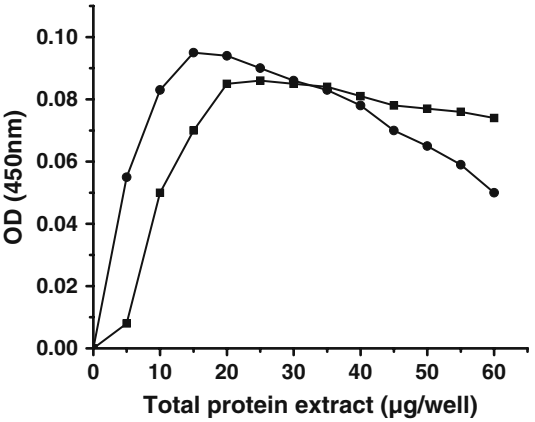

Fig. 13.7 ELISA test for the determination of the level of pp38 in sponge tissue extracts. For this series of experiments extracts from primmorphs had been used. ELISA plates were coated with 5–60 µg/well of sponge protein extract. Subsequently, the level of pp38 in the extract was detected with the commercial polyclonal antibodies against phosphorylated p38 (pp38) from Santa Cruz Biotechnology (■) and the anti-pp38 antibodies directed against the *S. domuncula* peptide (●) (Müller et al. 2002). Immunocomplexes were obtained by addition of a goat anti-rabbit IgG, which had been conjugated with horseradish peroxidase and visualized with the TMB substrate. Photometric determination was performed at 450 nm

from *S. domuncula* (Müller et al. 2002); this antibody was diluted 1:1000. In addition, a second, commercial polyclonal antibody which was raised against pp38 was used (Santa Cruz Biotechnology, California) and applied at a dilution of 1:2000. Again, the antibodies were diluted in PBS/BSA and incubated for 1 h. After blocking with 2% goat serum (Dianova, Hamburg, Germany) and 0.3% BSA in PBS for 30 min, the immunocomplexes were detected using goat anti-rabbit IgG, conjugated with horseradish peroxidase (1:10000; Sigma, Deisenhofen, Germany) under application of the 3,3,5,5-tetramethylbenzidine [TMB] substrate (KPL, Germany). The reaction was stopped after an appropriate time (typically 10 min) by adding 1 M H_2SO_4; the absorbance (OD_{450}) was measured at 450 nm (Titertek MultiScan Plus).

This direct ELISA assay was used to determine the p38 activation [phosphorylation] level in sponge primmorphs exposed to LPS. At first, the optimal concentration of the extract which reveals the highest signal in the photometric test was titrated. There sponge protein extract was added to the wells in concentrations between 5 and 60 µg/well of sponge protein extract (Fig. 13.7). The results revealed that at a concentration between 10 and 20 µg/well the highest OD_{450} can be recorded. Interestingly, the two antibodies used for this series of experiments, the commercial anti-pp38 (Santa Cruz Biotechnology, California) and our anti-pp38 raised against the *S. domuncula* peptide gave the same results.

Subsequently, this ELISA assay was applied to determine in a time-kinetic experiment the time required for the sponges to react to LPS. Primmorphs were exposed to 10 µg/ml of LPS for 5 min to 24 h. Then protein was prepared and subjected to

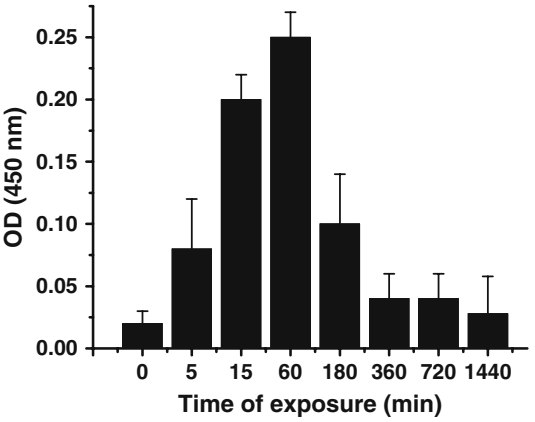

Fig. 13.8 Effect of LPS exposure on activation of pp38 in *S. domuncula* primmorphs. The tissue-like 3D-cell culture had been incubated for 5 min to 1440 min (24 h) with 10 µg/ml of LPS. Then protein was extracted and an aliquot of 20 µg was used for coating per well; then incubation with anti-pp38 (Santa Cruz Biotechnology) followed. The background values, measured with p38 antibodies, were subtracted

the ELISA assay to quantify the level of pp38. The highest level of phosphorylation of p38 (to pp38) was be measured after 60 min (Fig. 13.8).

From these data we conclude (i) firstly that primmorphs from *S. domuncula* respond – also under the experimental conditions used – to the exposure to LPS and (ii) secondly that the highest level of phosphorylation (formation of pp38) is seen after an exposure time of 60 min.

13.3.3 Real-Time Reverse Transcription-PCR (Method)

The technique of real-time reverse transcription-PCR (RT-qPCR) was used to quantify the expression levels of the genes coding for; (i) STEM CELL NUMBER: noggin (noggin-l [*S. domuncula*] NOGG-l_SD; accession number CAD59735; Müller et al. 2003b); (ii) PROLIFERATION: thymidine kinase (THYMKI_SD [*S. domuncula*]; accession number AM905441), (iii) DIFFERENTIATION: integrin-ß (integrin beta subunit [*S. domuncula*] INTb_SD; CAB38100; Wimmer et al. 1999a,b), (iv) LPS-CHALLENGE: perforin (macrophage expressed protein [*S. domuncula*] MPEG_SD; AJ890501; Wiens et al. 2005) and finally (v) THE HOUSEKEEPING GENE: glycerol 3-phosphate dehydrogenase (GAPDH_SD [*S. domuncula*]; accession number AM902265).

13.3.3.1 Total RNA Extraction

Samples were extracted using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Total RNA from primmorphs was isolated by the modified guanidine hydrochloride method (Yoshikawa et al. 2000). The concentration of each RNA sample was measured using a spectrophotometer. Only the RNA samples with a 260/280 ratio between 1.8 and 2.2 and a 260/230 ratio

higher than 1.9 were used for the analysis. In addition the integrity of RNA samples was assessed by agarose gel electrophoresis.

One microgram of each mRNA was reversely transcribed using the oligo dT primer and Super Script II (Invitrogen) as described (Yoshikawa et al. 2000).

13.3.3.2 Primer Design

For GAPDH expression studies the following primer pair had been selected: the forward primer was 5-ATCACAGGGGGAGCAAAGAA GGTCAT-3 [26mer] and the reverse primer 5-AAGTGGGGCTAGGC AGTTTGTGGTG-3 [25mer]; for *noggin* expression forward 5-TTCCCTCG ATATTTTTCTGCTGGCTCTT-3 [28mer] and reverse primer 5-CGTCCTTCCTCTTC GTCCTCTTACTATTG-3 [29mer]; for *integrin-ß* expression forward 5-CTTTGGACCTGCTTGCGAGTGTGA-3 [24mer] and reverse primer TCGCAA GCCGTTCCAAAGTAAGGTT-3 [25mer]; for *thymidine kinase* expression forward 5-CTTTCCCGATATTGTAGACTTTTG-3 [24mer] and reverse primer 5-CACACCATACAGACTGCCTTTAG-3 [23mer] and for *perforin* expression forward 5-CAATGAGATGTCCGGGTGGGTTTACT-3 [26mer] and reverse primer 5-GTTCTTTCGCAGGGTGGGCTTAGG-3 [24mer]. The resulting PCR product lengths were *GAPDH* fragment 150 bp [ranging from: 399-424 to 548-524]; *noggin* fragment 183 bp [422-449 to 604-576]; *integrin-ß* fragment 147 bp [1398-1421 to 1544-1520]; *thymidine kinase* fragment 165 bp [327-350 to 491-469] and *perforin* fragment 155 bp [1895-1920 to 2049-2026].

13.3.3.3 qPCR

RT-qPCR was done using the Light Cycler (Roche Diagnostics, Meylan, France), which exploits the ability of SYBR green to fluoresce after hybridization with a double-strand DNA. The analyses were performed in 20 AL glass capillaries using the Light Cycler fast start DNA master SYBR green kit (Roche Diagnostics). Then, 1 mM of each primer and 3 mM of $MgCl_2$ in the total volume of 20 µl were used in each real-time RT-PCR amplification. The real-time RT-PCR cycle started with the initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 57°C for 10 s for *GAPDH*, 56°C for 10 s for *noggin*, 57°C for 10 s for *integrin-ß*, 53.0°C for 10 s for *thymidine kinase*, and 57°C for 10 s for *perforin* and finally an elongation at 72°C for 10 s. As an internal quantitative control of the gene expression, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was determined.

13.4 Assessment of the Stem Cell Level in Primmorphs

It is known from earlier studies that primmorphs contain proliferating and differentiating cells (Custodio et al. 1998; Müller et al. 1999a). If these 3D-cell aggregates remain under slight rotation, they do not attach to the substratum (petri dish).

However, if they are placed onto coated plastic dishes (Wiens et al. 2003; Adell et al. 2007) they adhere and start to reconstitute to organ-like assemblies, expressing e.g. genes encoding proteins of the Wnt pathway.

13.4.1 Primmorph Formation

Primmorphs were obtained from single cells as described before (Custodio et al. 1998; Müller et al. 1999a); they reached sizes of 3–5 mm after three days. During this period the cultures were slightly shaken. Then, the primmorphs were transferred into a culture dish and cultivated further in natural seawater, supplemented with 0.2% of RPMI1640 – medium and the optimal silicate concentration (60 μM; Wiens et al. 2004). Incubation of the cultures continued for 1 to 3 days as follows; the primmorphs (i) remained under shaking in this medium in non-coated dishes, or (ii) were transferred into plates coated with recombinant *S. domuncula* galectin (Wiens et al. 2003). Primmorphs kept under such conditions strongly increased the proliferation and differentiation capacity of their cells. Among the genes which underwent high expression were the homeobox genes (Wiens et al. 2003). In the final series of experiments, the primmorphs (iii) were transferred to galectin-coated plates and incubated for 3 to 5 consecutive days with 10 μg/ml of LPS (Sigma L2880; LPS from *E. coli*). Representative images are shown in Fig. 13.4. Those primmorphs which remained under shaking for 3 days remained as ball-like aggregates in the medium, and did not attach to the substrate (Fig. 13.4E). Those primmorphs which could attach to the petri dish on the galectin coat, formed canal-like structures (Fig. 13.4F); and finally the primmorphs which had been incubated for 3 days in the presence of LPS developed apoptotic bodies (Fig. 13.4G).

13.4.2 Expression Pattern in Primmorphs for Noggin, Thymidine Kinase, Integrin and Perforin in Dependence on Adhesion and LPS Treatment

The primmorphs remained either untreated (formation of ball-like, non-adherent 3D-cell aggregates; Fig. 13.4E), or were placed onto galectin-coated culture dishes (attached primmorphs, which show canal-like structures Fig. 13.4F), or were treated with LPS (primmorphs which show “apoptotic” structures; Fig. 13.4G). Since the effect of the different treatments on gene expression should be measured, their duration was set to 1 and 3 days, respectively. After this incubation period the primmorphs were collected, RNA extracted, mRNA purified and finally analyzed by RT-qPCR for the transcript levels. In Table 13.1, the relative expression levels for the different marker genes (*noggin*, *thymidine kinase*, *integrin-ß*, and *perforin*) are summarized.

Table 13.1 Relative quantification of noggin, thymidine kinase, integrin-ß, and perforin in primmorphs under different treatment. The 3D-cell aggregates remained either non-attached (non-treated [culture dishes on a moving platform]), or cultured on galectin-coated dishes (attachment of the primmorphs and formation of canal-like structures), or were treated with LPS (formation of apoptotic bodies). The relative expression of reference genes in primmorphs is given. Assays of transcript levels for each reference gene were normalized by use of the housekeeping gene GAPDH. Each experiment has been performed 5-times; the means and the standard deviations are given

	Duration (days)		qPCR ratio [ratios × 100]			
Primmorphs Treatment:	1	3	Noggin: GAPDH	TK: GAPDH	Integrin-ß: GAPDH	Perforin: GAPDH
None	1		0.025 ± 0.003 [2.5-]	0.051 ± 0.006 [5.1-]	0.018 ± 0.004 [1.8-]	0.001 ± 0.001 [0.1-]
		3	0.031 ± 0.004 [3.1-]	0.047 ± 0.005 [4.7-]	0.024 ± 0.003 [2.4-]	0.003 ± 0.001 [0.3-]
Galectin-coated dishes	1		0.019 ± 0.004 [1.9-]	0.041 ± 0.005 [4.1-]	0.068 ± 0.007 [6.8-]	0.002 ± 0.001 [0.2-]
		3	0.017 ± 0.004 [1.7-]	0.039 ± 0.005 [3.9-]	0.072 ± 0.008 [7.2-]	0.0017 ± 0.002 [0.2-]
LPS	1		0.003 ± 0.001 [0.3-]	0.0009 ± 0.001 [0.1-]	0.0044 ± 0.005 [0.4-]	0.056 ± 0.007 [5.6-]
		3	0.007 ± 0.001 [0.7-]	0.001 ± 0.001 [0.1-]	0.0063 ± 0.007 [0.6-]	0.089 ± 0.010 [8.9-]

13.4.2.1 Expression in Non-treated Primmorphs

The highest expression level of the reference gene in primmorphs which remained in the rotating culture dishes was seen for *noggin*; with a ratio to *GAPDH* of 0.025 [2.5-fold (×100) expression with respect to *GAPDH*; 1 day incubation period] (Table 13.1). This value increased to 3.1-fold after 3 days. Comparably high is – in these primmorphs – also the expression level of the *thymidine kinase*; a 5-1-fold ratio has been measured at day 1, and a 4.7-fold at day 3. A lower level had been determined for *integrin-ß* with 1.8- and 2.4-fold (day 1 and day 3, respectively). Very low is the transcript level of *perforin*; it measures at day 1 0.1-fold and at day 3 0.3-fold.

13.4.2.2 Expression Levels in Attached Primmorphs

As expected, the relative level of *integrin-ß* expression is highest with 6.8-fold (day 1) and 7.2-fold (day 3); Table 13.1. Comparable with the ratios in the non-attached primmorphs are the values for *thymidine kinase* with 4.1-fold (day 1) and 3.9-fold (day 3); lower is the ratio for *noggin* with 1.9-fold (day 1) and 1.7-fold (day 3). The expression ratio for *perforin* 0.2-fold (remained unchanged).

13.4.2.3 Level of Expression in LPS-Treated Primmorphs

A drastic effect on the expression level is seen when primmorphs are incubated with LPS (Table 13.1). The ratios of expression for *noggin*, *thymidine kinase* and

integrin-ß drop to very low levels, with 0.3-fold (*noggin*), 0.01-fold (*thymidine kinase*) and 0.4 (*integrin-ß*). In contrast, the level for perforin expression strongly increases to 5.6-fold (day 1) and 8.9-fold (day 3) in LPS treated primmorphs.

13.5 Effect of LPS Treatment on the Number of Stem Cells

Exposure of mouse embryo stem cells to LPS results in Fas-mediated apoptosis (Zou et al. 2000). On the other hand, it had been reported that LPS causes an induction of mesenchymal stem cells towards an osteogenic differentiation (Cho et al. 2006). Therefore, it was suggestive to study the effect of the endotoxin LPS on the number of stem cells in the primmorph system.

In order to provide the basis for conclusive results, the number of stem cells in non-differentiated (ball-like non-attached primmorphs) and substrate-caused differentiating primmorphs (attached aggregates) had to be assessed first. To estimate the number of stem cells, the level of expression of noggin (marker for toti-/omni-potent cells) had been quantified by qPCR. In parallel, the transcript level of thymidine kinase (marker for proliferation), integrin-ß (marker for substrate adhesion) and perforin (marker for the anti-bacterial effector protein) had been determined.

In the absence of any additional extracellular effector/mediator the ball-like primmorphs show a high level of expression of *noggin* and *thymidine kinase* genes (Table 13.1) This result confirmed earlier observations that cells in these aggregates show a high proliferation capacity – and no apparent sign of formation of tissue-like assemblies (Custodio et al. 1998; Müller et al. 1999a). A schematic representation is given in Fig. 13.9.

The primmorphs which had been attached to the substrate, the galectin-coated culture dishes, show a high expression of the gene encoding integrin, and considerable levels of *thymidine kinase* and *noggin* transcript (Table 13.1), while the expression of perforin is low (Fig. 13.10).

Based on the recent observation, suggesting that the signal transduction pathway, originating from pattern-recognition receptors, activates also the MAP-kinase system (Lee and Kim 2007; Beisswenger et al. 2007), we investigated whether LPS exposure results in a differential alteration of gene expression in primmorphs. Indeed, it was determined that the expression level of the defense molecule against microorganisms, perforin, strongly increases from 0.1-fold (untreated primmorphs) to 5.6-fold (LPS-exposed primmorphs); Table 13.1. This upregulation is selective, since simultaneously the expression level of *noggin*, *thymidine kinase* and *integrin* undergo a drastic reduction (Table 13.1; Fig. 13.11). It remains to be studied if this effect is reversible. Considering these data – positive impact of anti-bacterial proteins on gene expression under simultaneous down-regulation of expression of those genes which regulate differentiation plasticity and cell differentiation – support the view that the LPS caused effect on cell metabolism results also in an induction of apoptotic signaling pathways.

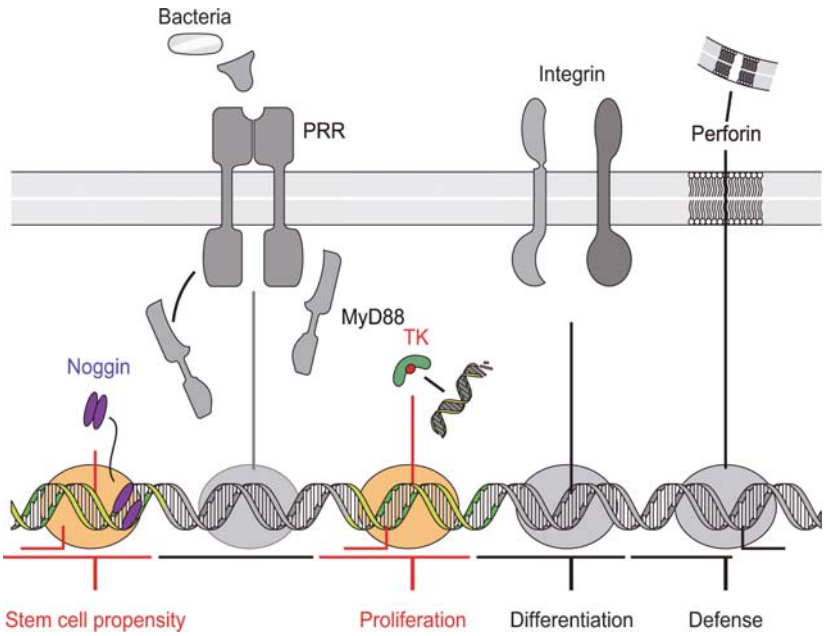

Fig. 13.9 Schematic outline of the signal-transduction pathways and of the gene expression levels in primmorphs which remained in a non-attached state in the culture dish. In these aggregates the cell-surface associated receptors, pattern-recognition receptor (PRR) and the integrins, are (if at all) only insignificantly expressed. Consequently, the underlying genes, *pattern-recognition receptor* and *integrin*, is minimal. Likewise the gene encoding the executing extracellular attacking protein, perforin, is not expressed. In contrast, the transcript level of *noggin* (an indicator for the number of stem cells) and *thymidine kinase* is high, indicating a substantial level of cells that are provided with high differentiation plasticity and proliferation capacity

13.6 Concluding Remarks

There was never a faster progress in the understanding of the differentiation capacity of sponge cells than during the last 10 years when sophisticated molecular biological techniques were developed and applied. It became possible to trace back empirical observations on a causal-analytical basis. Already over 100 years ago the astonishing regenerative power of sponge cells (Schmidt 1862), and of archaeocytes in particular (Wilson 1907), had been recognized. The concept of embryonic unspecialized cells in hexactinellids had been introduced by Schulze (1904) who coined them “sorites”. These sorites were described as separate cell types besides the germ cells. The processes of spermatogenesis, oogenesis and embryogenesis of Demospongiae had been described in detail by Noll (1888). Interesting – but at the time not recognized – are the contributions of Diaz (1979) on the transformation of cells in sponges through differentiation and de-differentiation. Especially his view on the plastic differentiation capacity between archaeocytes and

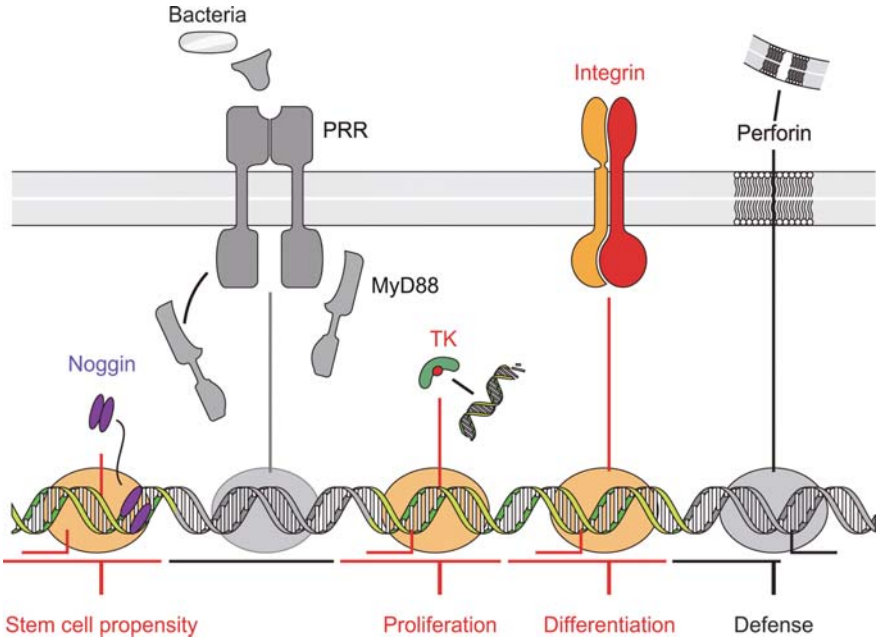

Fig. 13.10 Expression level in primmorphs which had been attached to galectin-coated petri dishes. The *integrin* transcript level is high and also the level for *noggin* and *thymidine kinase* is substantial. Again, the amount of transcripts encoding the toxic protein perforin are low

epithelial cells is here relevant. After the identification of the first protein-coding genes in *G. cydonium* and *S. domuncula* it became suddenly overt that sequences of sponge genes are metazoan-like and share high sequence similarity to those found in humans (Pfeifer et al. 1993). In addition, the *in vitro* primmorph cell culture system became a powerful tool to study gene expression in "embryonic" cells.

Focusing on the stem cell system in sponges the main lessons are; (i) their cells progress from a primordial stage to terminally differentiated stages, (ii) they contain totipotent stem cells, (iii) during the progression from stem cells to differentiated cells genes are expressed among which some share high sequence similarity to those identified in vertebrates. At present the prevailing notion recognizes the high plasticity of stem cells because of the high regeneration/repair capacity of somatic sponge cells. Molecular genetic studies in sponges will continue to assess this view. After all, it is expected that ultimately sponges become the model (Pilcher 2005). Finally it could be demonstrated that an exposure of primmorphs to LPS causes a reduction of the number of stem cells under simultaneous upregulation of the defense molecule perforin.

Note: The following sequences from *Suberites domuncula* have been deposited (EMBL/GenBank): the cDNA for the glycerol 3-phosphate dehydrogenase

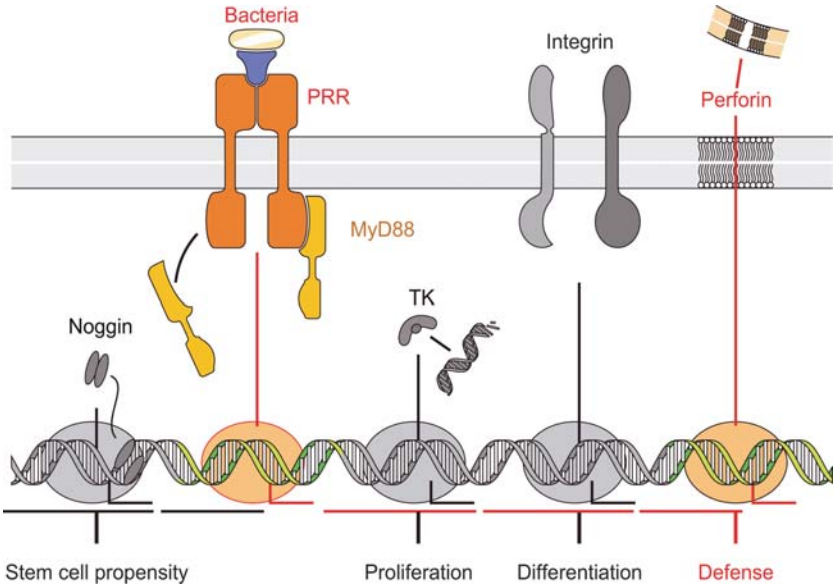

Fig. 13.11 Innate immune response in sponges against Gram-negative bacteria. The simplified model of the LPS-mediated signaling shows that LPS, a pathogen-associated molecular pattern (PAMP), causes (homo)dimerization of the LPS-binding protein, a pattern-recognition receptor (PRR), on the cell surface. After complex formation between PAMP and PRR, PRR initiates a signal transduction cascade, with MyD88 as the first member. Downstream, intermediate members of the signaling pathway, which likely involves IRAKs, TRAFs, NF-κB and also LITAF (to be published), transduce an increased expression of the activation phase's key molecules, PRR and MyD88, and finally of the executing effector/defense molecule, the perforin-like protein

(GAPDH_SD) under AM902265 and for thymidine kinase (THYMKI_SD) under AM905441.

Acknowledgments This work was supported by a grant from the Deutsche Forschungsgemeinschaft (WI 2116/2-2), the Bundesministerium für Bildung und Forschung (project C1 20 20: cooperation project WTZ BRA – Health of marine ecosystems (03F0451A), and Center of Excellence *BIOTEC*marin) and the International Human Frontier Science Program (RG-333/ 96-M).

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