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ScienceDirect
Micron 39 (2008) 447–460
micron
www.elsevier.com/locate/micron

Regional and modular expression of morphogenetic factors in the demosponge *Lubomirskia baicalensis*☆

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Received 8 January 2007; received in revised form 7 February 2007; accepted 8 February 2007

Abstract

Some sponges [phylum Porifera], e.g. the demosponges *Lubomirskia baicalensis* or *Axinella polypoides*, show an arborescent growth form. In the freshwater sponge *L. baicalensis* this morphotype is seen mostly in depths below 4 m while in more shallow regions it grows as a crust. The different growth forms are determined in nature very likely by water current and/or light. The branches of this species are composed of modules, arranged along the apical–basal axis. The modules are delimited by a precise architecture of the spicule bundles; longitudinal bundles originate from the apex of the earlier module, while at the basis of each module these bundles are cross-linked by traverse bundles under formation of annuli. Genes encoding putative morphogenetic factors, myotrophin and epidermal growth factor (EGF)-like molecules, and one gene of an antagonist for the Wnt signaling pathway, the soluble frizzled molecule, have been identified and characterized. Their expression levels as well as those of silicatein, one major spicule-forming molecule, have been studied in the crusts and the modules. The data revealed that at the apices of each module higher level of expression of *myotrophin* and *EGF* can be detected, while the base of each module is characterized by a high steady-state expression level of *soluble frizzled molecule*. These results suggest that module formation in *L. baicalensis* is controlled by a tuned interaction of agonistic (e.g., myotrophin and EGF) as well as antagonistic morphogenetic factors (e.g., soluble frizzled molecule).

Keywords: Sponges; Porifera; Body plan; Axis formation; *Lubomirskia baicalensis*; Myotrophin; Epidermal growth factor; Soluble frizzled molecule

1. Introduction

Sponges [phylum Porifera] are the simplest metazoans. Data on sponge genes and their deduced proteins, from the demosponge *Suberites domuncula*, as well as their functional analyses revealed that the taxon sponges branched off first from the hypothetical common ancestor of all Metazoa, the Urmetazoa (Müller, 2001; Pilcher, 2005). Surprisingly, these studies disclosed also that sponges comprise already a functional (natural) immune system (Müller et al., 1999a). The efficiency of the host defense system is supported by apoptotic protection mechanisms allowing sponges to undergo controlled cell death in response to histoincompatibility reactions (Müller et al., 2002) or adverse environmental effects (Wiens et al., 2001). The individuality of sponges, as proposed by Hartman and Reiswig (1973), was not only supported by immunological studies but also by recent data on the role of genes involved in morphogenesis (Wiens et al., 2003) and axis formation (Adell et al., 2003; Adell and Müller, 2005). In adult sponge specimens, the skeleton-forming proteins, e.g. silicatein, provide the matrix for spicule formation in particular and axis formation in general (Müller, 2005). During the proposed gastrulation, sponge larvae develop a polarity (Vosmaer, 1887;

☆ *Note*: The sequences from *Lubomirskia baicalensis* reported here, the *soluble frizzled molecule* (*LBSFRP*; accession number AM231311), the *myotrophin* (*LBMYOL*; AM231309) and the *epidermal growth factor-like precursor protein* (*LBEGFl-PREC*; AM231310) are deposited in the EMBL/GenBank database.

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URL: http://www.biotecmarin.de/

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doi:10.1016/j.micron.2007.02.003

Maldonado, 2004; Leys, 2004), a characteristic that has been lost in some sponge species at later stages. Recent findings on the presence and function of homeobox genes, especially those coding for LIM/homeodomain transcription factors (Wiens et al., 2003), led to the hypothesis that sponges are provided with a genetically controlled body plan (Müller et al., 2004), which allows the formation of an architectural pattern with one main apical–basal body axis (Müller, 2005).

Adult specimens of the endemic freshwater sponge *Lubomirskia baicalensis* display varying morphologies, from lumpish massive irregular crusts to arborescent/fan-shaped or dichotomously branching animals (Dybowsky, 1880; Kozhov, 1963; Wiens et al., 2006). This species propagates sexually through frequently formed larvae (Annandale, 1914). Histological (Masuda et al., 1997) and structural studies (Müller et al., 2006a) revealed the highly ordered arrangement of the spicules (curved/fusiform amphioxea) forming the skeleton of this species into longitudinal bundles, which are connected by cross bundles. The spicules are embedded into a bulky organic matrix; in the average about 5–7 spicules form the inorganic central core of the bundles. Characteristically, 1–2.5 cm long septate modules are arranged along the growing axis of the branches. Based upon expression studies of genes encoding skeletal proteins, silicatein and mannose-binding lectin, as well as of mago nashi, initial experimental evidence had supported the notion that gene expression gradients exist along the branches of *L. baicalensis* (Wiens et al., 2006).

Skeletogenesis is one major morphogenetic prerequisite for growth. In siliceous sponges (Demospongiae and Hexactinellida) the skeletal elements are spicules built of inorganic silica (see Perry, 2003; Pisera, 2003). The exceptional feature of spicule formation is that biosilica is synthesized in sponges enzymatically by silicatein (Shimizu et al., 1998). Since the shape of the amazingly diverse spicules varies species-specifically (see Uriz et al., 2003), a tuned expression of silicatein will crucially contribute to the construction of the body plan. Amazingly enough the freshwater sponge *L. baicalensis* contains not just two silicatein genes, like marine sponges; thus far, at least four different silicateins are known to be present (Wiens et al., 2006).

In the present study, we isolated genes from *L. baicalensis* which are known to be involved in cell-fate decisions, tissue polarity and morphogenesis. In marine sponges, e.g. in *S. domuncula*, frizzled is expressed in the epidermal layer(s), in regions which are rich in stem cells (Adell et al., 2003). First, the frizzled protein is described; it comprises the frizzled domain but neither the transmembrane domain nor the Lys-Thr-X-X-X-Trp motif, found in frizzled receptors for signaling (Umbhauer et al., 2000). Hence, the *L. baicalensis* frizzled molecule belongs to the class of secreted frizzled proteins that act as antagonists of the Wnt signaling pathway (Leyns et al., 1997). In the canonical Wnt signaling pathway, the activated frizzled receptor binds to Dishevelled (Dsh), which leads to the stabilization and accumulation of β-catenin in the nucleus, where it activates the TCF/LEF transcription factor (reviewed in Cadigan and Nusse, 1997; McEwen and Pfeifer, 2000). Recently, a series of molecules involved in the Wnt pathway of *S. domuncula* have been identified (Adell, in preparation). The second molecule described here, myotrophin, is known to be highly expressed within the sponge *S. domuncula* in areas that are characterized by a high proliferation and differentiation capacity (Schröder et al., 2000; Krasko et al., 2000). Myotrophin had originally been identified in mammalian cardiac tissue undergoing hypertrophy (see Sil et al., 1998); it comprises two protein-protein binding domains. In *S. domuncula*, recombinant myotrophin was found to cause a dose-dependent increase in protein biosynthesis *in vitro* (Schröder et al., 2000). These data revealed that within the concentration range of 0.1–10 μg/ml this polypeptide causes a 10-fold increase in the incorporation rate of [3H]Lys into the acid-insoluble fraction, during an incubation period of 12 h (Schröder et al., 2000). In the present study, this sponge molecule was tested *in vitro* on the protein synthesis using primmorphs from *L. baicalensis*. Primmorphs are three-dimensional cell aggregates, containing proliferating and differentiating cells (Müller et al., 1999b). Finally, the cDNA for a cytokine, the epidermal growth factor [EGF] (Kataoka et al., 2002), was isolated from *L. baicalensis*. EGF had previously been shown to be induced in other sponges in areas of high proliferation activity (Perović-Ottstadt et al., 2004). In general, EGF plays an important role in the regulation of cell growth, proliferation, and differentiation.

The expression of these three genes of *L. baicalensis*, coding for morphogenetic factors, was determined in the uppermost two to three modules of a branch. In addition, the expression of the silicatein-α1 gene (Wiens et al., 2006) was investigated to define its (potential) role in the regionalization within one module. This gene was found to be most differentially expressed along the axis (Wiens et al., 2006); the expression at the top of the branch is very high, while almost no expression is seen at its base.

2. Materials and methods

2.1. Chemicals and enzymes

Restriction enzymes, SNAP ''Total RNA Isolation Kit'', Superscript II and reagents for RACE procedure were purchased from Invitrogen (Carlsbad, CA, USA); *TriplEx2* vector from BD (Palo Alto, CA, USA); TRIzol Reagent from GibcoBRL (Grand Island, NY, USA); Hybond-N^+ nylon membrane and L-[4,5-^{3}H] lysine (Lys; specific activity of 7.3 Ci/mmole) from Amersham (Little Chalfont, Buckinghamshire, UK); DAPI [(4′,6-diamidino-2-phenyindole] from Sigma (St. Louis, MO, USA). PCR-DIG-Probe-Synthesis Kit and CDP from Roche (Mannheim, Germany); Lake Baikal water was obtained from ''Lake'' Comp (Irkutsk, Russia).

2.2. Sponges and cDNA library

Specimens of *L. baicalensis* (Porifera, Demospongiae, Haplosclerida) were collected in Lake Baikal (Russia) near the village Listvianka from depths between 2 and 12 m. The