Polarity factor 'Frizzled' in the demosponge *Suberites domuncula*: identification, expression and localization of the receptor in the epithelium/pinacoderm[1]

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Abstract Until recently, it was assumed that polarity and axis formation have evolved only in metazoan phyla higher than Cnidaria. One key molecule involved in the signal transduction causing tissue polarity is Frizzled, a seven-transmembrane receptor that is activated by the Wnt family of secreted proteins. We report the isolation and characterization of a *Frizzled* gene from the demosponge *Suberites domuncula* (*Sd-Fz*). The deduced polypeptide comprises all characteristic domains known from Frizzled receptors of higher metazoans. In situ hybridization studies show that *Sd-Fz* is expressed in cells close to the surface of the sponges and in the pinacocytes of some canals. Northern blot analysis demonstrates its upregulation during the formation of three-dimensional sponge cell aggregates in culture. These data provide for the first time experimental evidence that already in the lowest metazoan phylum (Porifera) genes are present which are very likely involved in tissue polarity.

Key words: Sponge; Porifera; Frizzled receptor; Tissue polarity; Axis formation; *Suberites domuncula*

1. Introduction

Molecular and cell biological evidence has clarified in the last 10 years that sponges (phylum Porifera) share with the other metazoan phyla one common origin (see [1–3]); the hypothetical ancestor was termed Urmetazoa [4]. After this enigmatic question of phylogeny was answered, it was obvious to ask for the origin of a series of characteristic metazoan features. Among those, the common elements of the cell adhesion systems of the metazoan phyla, such as integrins and their interacting ligands, had been described [5,6]. Subsequently, the common basis of the metazoan immune system was defined after the discovery that a series of molecules regulating self–self and self–non-self recognition are conserved from sponges to human [7,8]. Recently, great progress has been made by establishing that the Porifera share with all other metazoans the genetic repertoire for a controlled cell fate determination and morphogenesis. Lim class homeodomain proteins [9], T-box transcription factors [10], and genes involved in apoptosis [11] have been isolated and characterized in Porifera. Even *noggin*, a gene typical of the Spemann organizer of higher metazoan phyla, has recently been reported [12].

The Wnt signaling pathway is a cell communication system which regulates cell fate decisions, tissue polarity and morphogenesis (reviewed in [13–15]). The Frizzled protein is the membrane receptor for the Wnt secreted glycoproteins. Its molecular structure comprises a large cysteine-rich extracellular domain, a seven-transmembrane spanning domain and a short cytoplasmic tail. Through the canonical Wnt signaling pathway the activated Frizzled binds to Dishevelled (Dsh), which leads to the stabilization and accumulation of β-catenin in the nucleus; there it activates the TCF/LEF transcription factor (reviewed in [13,14,16]).

In the present study we report the isolation of the Frizzled receptor from the demosponge *Suberites domuncula* (*Sd-Fz*). In situ hybridization analysis in adult specimens demonstrates its expression in the cortex region and in the epithelial layer of the aquiferous canals. Northern blot analysis reveals an upregulation of its levels of expression during the formation of sponge cell aggregates (primmorphs). These results strongly suggest that the Wnt pathway is already present in Porifera.

2. Materials and methods

2.1. Chemicals and enzymes

The sources of chemicals and enzymes used have been given previously [17,18].

2.2. Sponges

Specimens of *S. domuncula* (Demospongia, Hadromerida, Suberitidae) were collected from the Adriatic Sea, close to Rovinj (Croatia), and then kept in aquaria in Mainz for over 3 years, as described by Le Pennec et al. [19].

2.3. Dissociation of cells and formation of primmorphs

The procedure described for the formation of primmorphs from single cells was applied [20,21]. Single cells were obtained by dissociation in Ca^{2+}- and Mg^{2+}-free artificial seawater (CMFSW [22]) and were plated in a plastic cell culture dish in artificial seawater [22], supplemented with 0.2% RPMI 1640 medium. Sponge cells first adhere to the substrate (now termed adhered aggregates); after the second day they spontaneously detach and after approximately 5 days, 3–7 mm large primmorphs form.

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[1] The sequence from *Suberites domuncula* reported here, Frizzled receptor, is deposited in the EMBL/GenBank data base under the accession number AJ566637.

Abbreviations: CMFSW, Ca^{2+}- and Mg^{2+}-free artificial seawater; PBS, phosphate-buffered saline; PCR, polymerase chain reaction

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2.4. Isolation of a cDNA for the Frizzled receptor

As a result of a *S. domuncula* cDNA sequencing project that is being performed in our laboratory, a fragment of a cDNA encoding part of the transmembrane domain of a putative Frizzled receptor was isolated. Using the rapid amplification of cDNA ends technique (Invitrogen GeneRacer Kit; Invitrogen, Groningen, The Netherlands) a full-length cDNA of 1770 bp was obtained.

2.5. Phylogenetic analysis of the Frizzled receptor

The deduced amino acid sequence of Sd-Fz was compared with Frizzled receptors of other organisms by the neighbor-joining method [23]. The degree of support for internal branches was further assessed by bootstrapping [24]. Accurate multiple protein sequence alignments were made using the software CLUSTAL W [25].

2.6. Northern blotting

Samples of sponges living in the aquarium were immediately frozen, pulverized in liquid nitrogen and RNA was extracted using the TRIzol reagent (Gibco BRL, Grand Island, NY, USA). Primmorphs of different stages of culture were directly lysed and RNA was extracted in the TRIzol reagent. 8 μg of total RNA was fractionated by electrophoresis, transferred to a Hybond N^+ nylon membrane (Amersham, Buckinghamshire, UK), and hybridized overnight at 50°C. The probe, corresponding to the last 487 bp of the coding region of Sd-Fz, was labeled with the PCR DIG Probe synthesis kit (Roche, Mannheim, Germany). After washing digoxigenin (DIG)-labeled nucleic acid was detected with anti-DIG Fab fragments conjugated to alkaline phosphatase, and visualized by the chemiluminescence technique using CDP-star (Roche). The RNA concentration was measured by absorbance spectroscopy [26]. To quantitate the signals of the Northern blots the chemiluminescence procedure was applied [27].

2.7. In situ localization studies

The method applied is based on the procedure described by Polak and McGee [28] with modifications described recently [29]. 8 μm frozen sections were obtained at −30°C using a Slee cryostat (Mainz, Germany). Cryosections were fixed with paraformaldehyde (4% in 1× phosphate-buffered saline, PBS), washed twice with PBS, incubated for 15 min at room temperature with proteinase K and subsequently fixed again with paraformaldehyde. To remove the sponge color the sections were washed with increasing concentrations of ethanol (30–90%) followed by washes with decreasing concentrations of acetone (100–30%). After rehydration with decreasing concentrations of ethanol and a final wash with PBS, DIG-labeled DNA probes were added to the hybridization solution. Hybridization was performed overnight in a humid chamber at 42°C. Washes were performed at 52°C as described [29]. After blocking, the sections were incubated with an anti-DIG antibody conjugated with alkaline phosphatase. The dye reagent NBT/X-phosphate was used for visualization of the signals.

Antisense and sense ssDNA DIG-labeled probes, corresponding to the last 487 bp of the coding region of Sd-Fz, were synthesized by polymerase chain reaction (PCR) using the PCR DIG Probe synthesis kit (Roche). Sense probes were used in parallel as negative controls in the experiments.

2.8. Histological analysis

Primmorphs were fixed in 4% paraformaldehyde/PBS. After dehydration in ethanol, the samples were embedded in Technovit 8100 [30], according to the instructions of the manufacturer. Sections of 2 μm thickness were prepared and stained with Ziehl's fuchsin [31].

3. Results

3.1. Identification of Sd-Fz in S. domuncula

The cDNA encoding the complete Frizzled receptor, *Sd-Fz*, was isolated as described in Section 2. The nucleotide sequence comprises 1770 nucleotides, with an open reading frame predicting a protein of 529 amino acid residues. The alignment of Sd-Fz with Frizzled sequences from other animals (Fig. 1A) shows the conservation in Sd-Fz with the characteristic cysteine-rich extracellular domain (Frizzled domain), as well as the seven-transmembrane spanning domains. In its cytoplasmic region a conserved hexapeptide motif (K-T-X-X-X-W), which has been reported to interact with the Disheveled protein [32], is also present. A phylogenetic tree was constructed with the related sequences from the data banks, showing that Sd-Fz is located at the base of the metazoan Frizzled receptors (Fig. 1B).

3.2. Identification of cells expressing Frizzled receptor

Sections from *S. domuncula* were analyzed by in situ hybridization to identify those cells expressing the Frizzled receptor. Cells within a 50–100 μm wide rim at and immediately under the surface of the animals are brightly stained (Fig. 2A,B). Likewise, the pinacocytes of some canals are intensely stained. A higher magnification of a cross-section through a canal shows a single or double cell layer of the pinacoderm positive for *Sd-Fz* (Fig. 2C). No cells were stained when the sense strand was used as a probe (Fig. 2D).

3.3. Expression of Frizzled receptor in S. domuncula *cell cultures*

Tissue from two different sponges was dissociated into single cells in CMFSW, and then transferred to a cell culture plate containing Ca^{2+}- and Mg^{2+}-containing seawater. During the first 2 days the cells started to form three-dimensional cell aggregates adhered to the substrate. From the third day on the aggregates detached from the substrate and began to form primmorphs (three-dimensional cell aggregates in suspension) [19]. A cross-section through a primmorph shows that the surface is surrounded by a single or double cell layer of pinacocytes (data not shown). A schematic outline of primmorph formation is given in Fig. 3.

In order to determine if the expression of *Sd-Fz* might be regulated during the culture of the sponge cells, Northern blot analysis was performed. As seen in Fig. 4, the levels of expression of *Sd-Fz* in the sponge adhered aggregates (Fig. 4; AtA) and in primmorphs (Fig. 4; Pri) are upregulated (over five-fold) compared to that in sponge tissue (Fig. 4; An). Interestingly, the maximal level of expression of *Sd-Fz* was detected during the first day of culture, when sponge cells form the adhered aggregates.

4. Discussion

Frizzled proteins are seven-transmembrane receptors which bind the members of the secreted glycoproteins Wnt/Wingless family. The Wnt-Frizzled signaling pathway is a cell–cell communication system involved in the establishment of tissue polarity and cell fate determination. It is implicated in a wide range of developmental processes such as segmentation in *Drosophila*, asymmetrical divisions in *Caenorhabditis*, or the dorso-ventral axis specification and the patterning of the central nervous system in vertebrates [13,14,33,34]. The discovery of the *Frizzled* gene (*Sd-Fz*) in the demosponge *S. domuncula* came as a surprise. Even though Haeckel already in 1874 [35] grouped sponges with the 'Diblasteria', composed of two permanent germ layers, the 'Exoderma' and the 'Endoderma', this view was not accepted; today the distinction between Metazoa and Eumetazoa is still made on the grounds of histological data. In contrast to the other metazoan phyla sponges are considered not to have an epithelium because they are devoid of a basement membrane [36]. However, since

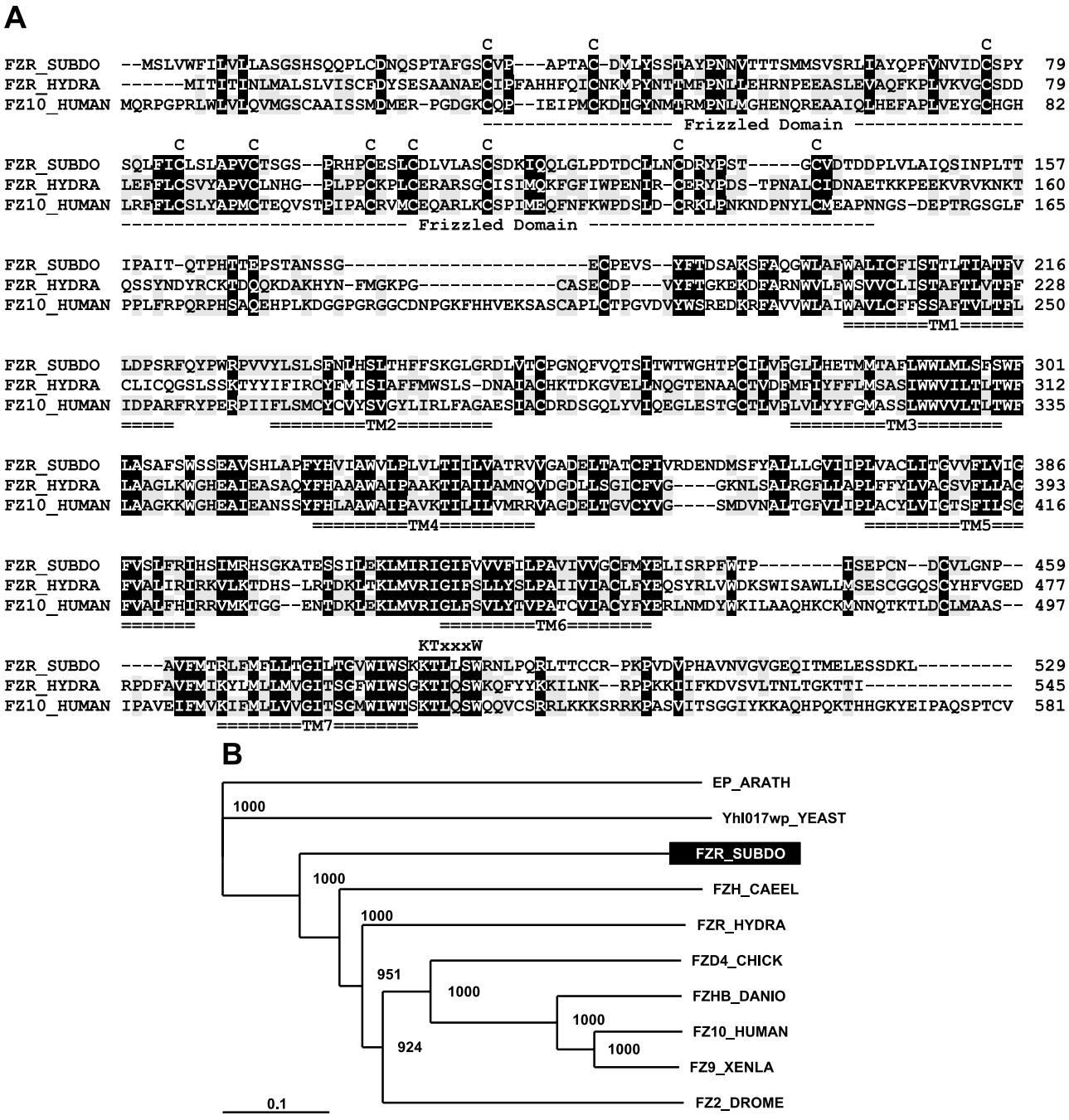

Fig. 1. A: Alignment of the sponge Frizzled receptor (Sd-Fz [FRZ_SUBDO]) with Frizzled molecules from *Hydra vulgaris* (FZR_HYDRA; AF209200) and human (FZ10_HUMAN; NP_009128). Amino acids similar among all sequences are in inverted type and those conserved in at least two sequences are shaded. The amino acid residues characteristic for the conserved Frizzled domain, the seven-transmembrane spanning domains (TM1–TM7) as well as the hexapeptide motif (K-T-X-X-X-W) are marked. B: Phylogenetic tree constructed from the above-mentioned sequences as well as the metazoan Frizzled from *Caenorhabditis elegans* (FZH_CAEEL; NP_492635), *Drosophila melanogaster* (FZ2_DROME; S71786), chicken (FZD4_CHICK; Q9IA05), fish (FZHB_DANIO; CAD10102), frog (FZ9_XENLA; AAD44332), as well as the distantly related polypeptides from *Arabidopsis thaliana* (EP_ARATH; NP_180589) and yeast (Yhl017wp_YEAST; NP_011846). After alignment the tree was built and rooted using the plant sequence as an outgroup. The numbers at the nodes are an indication of the level of confidence for the branches as determined by bootstrap analysis (1000 bootstrap replicates). Scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per position in the sequence.

the discovery of integrins [5] and fibronectin-related ligands [37], this assumption should be abandoned.

In the present report, the in situ hybridization study revealed that in adult sponges the cells that express *Sd-Fz* are located close to the surface of the specimens and also in the surrounding of the aquiferous canal system. This expression pattern is reminiscent of that known from the diploblasts [38], protostomia [39] and deuterostomia [40], where *Frizzled* is expressed in the germ layers which give rise to the ectoderm, endoderm and mesoderm. Experimental evidence indicates that the development of the aquiferous canal system in sponges starts by formation of tubes from the surface of the

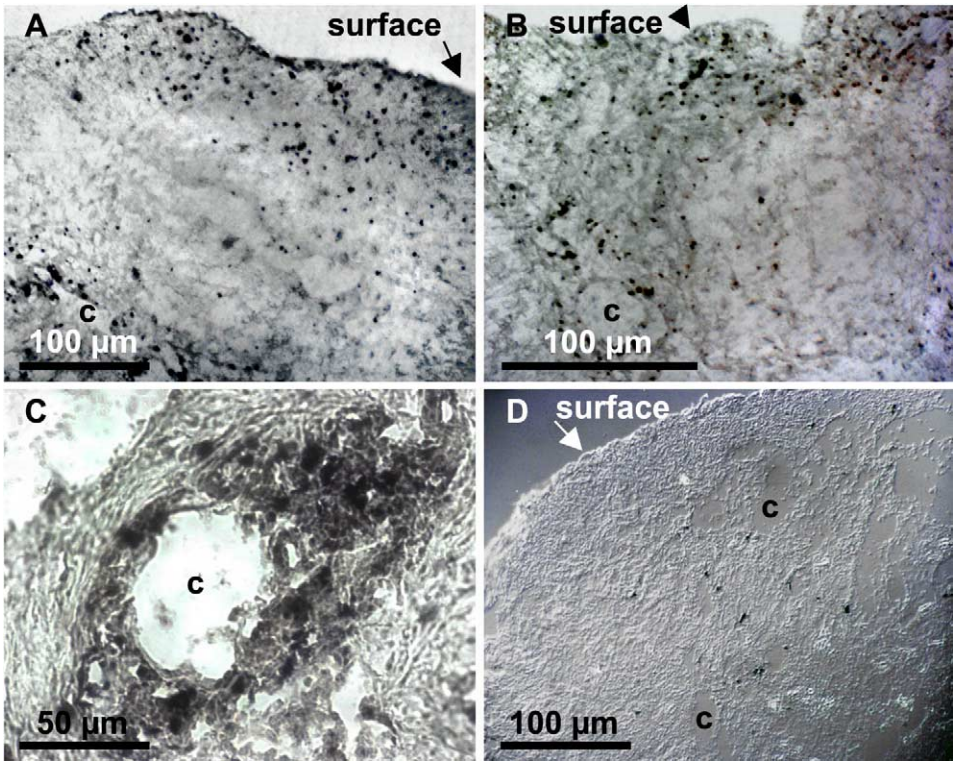

Fig. 2. Spatial expression pattern of Frizzled receptor in *S. domuncula*. Cryosections were performed from sponge tissue and hybridized with DIG-labeled *Sd-Fz* antisense (A–C) or sense (D) ssDNA probes. A, B and D correspond to sections showing the surface of the specimen. In A–D a section of a canal (c) is shown. The canals are lined by an epithelial layer formed of pinacocytes. The size markers are given.

animals, the porocytes, and by canals originating from the choanocyte chambers [41]. Therefore, the data presented here correlate the expression of *Sd-Fz* with the formation of canals in sponges.

In the sponge cell culture the totipotent dissociated sponge cells (archeocytes) attach to the substrate, and begin to form structured aggregates, which contain pinacocytes. After the third day the aggregates begin to detach now forming organized three-dimensional aggregates in suspension (primmorphs), which also contain differentiated pinacocytes in the epithelium covering the surface and the canals [21]. The up-regulation of *Sd-Fz* during the reorganization of the sponge tissue together with its pattern of expression in the adult animals suggest that a Wnt pathway involved in cell fate determination and morphogenesis was already established in the phylogenetically oldest extant metazoan.

There is evidence that in higher metazoans three different signaling pathways exist that are triggered by the Wnt-Frizzled interaction, which separately regulate cell fate determination, tissue polarity and cell adhesion. In the canonical Wnt pathway, the binding of Wnt to Frizzled leads to the inhibition of β-catenin degradation and its accumulation in the nucleus, where it activates the transcription of specific genes to control cell fate [13,32]. However, a β-catenin-independent pathway has also been extensively reported in processes such as the planar cell polarity in *Drosophila* and the convergent extension during gastrulation in vertebrates. In this alternative pathway the activation of Frizzled does not lead to a transcriptional regulation, but to the cytoskeletal changes needed for morphogenesis [15,16,42,43]. A third Wnt-Frizzled pathway induces the mobilization of intracellular Ca^{2+} and activates Ca^{2+}-dependent enzymes, such as protein kinase C [44]. How the different pathways are regulated and what determines their specificity is not clear. As in vertebrates several Wnt and Frizzled molecules have been characterized, it has been proposed that the particular combination of Wnt and Frizzled determines which pathway will be used [45,46]. In this context the elucidation of the molecules involved in the Wnt pathway in a simple animal such as a sponge, which is expected to have just one representative of

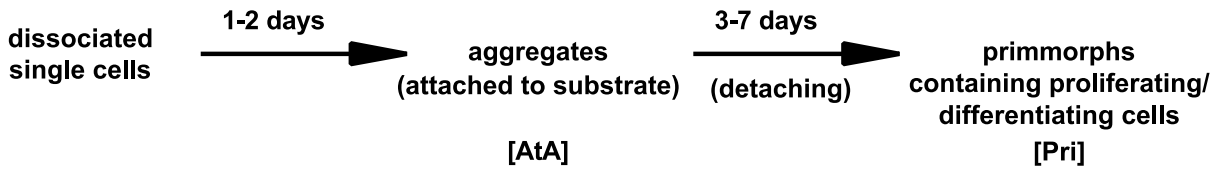

Fig. 3. Schematic outline of primmorph formation in *S. domuncula*. Single cells were obtained after dissociation of tissue in CMFSW. After transfer of the cells into Ca^{2+}- and Mg^{2+}-containing seawater, the adhered aggregates (AtA) are formed after 1–2 days. Continuing the incubation for at least 5 days leads to formation of primmorphs. Cross-sections through primmorphs (Pri) showed that they comprise proliferating and differentiating cells. Applying staining with Ziehl's fuchsin showed that the primmorphs are surrounded by a single or double cell layer of pinacocytes.

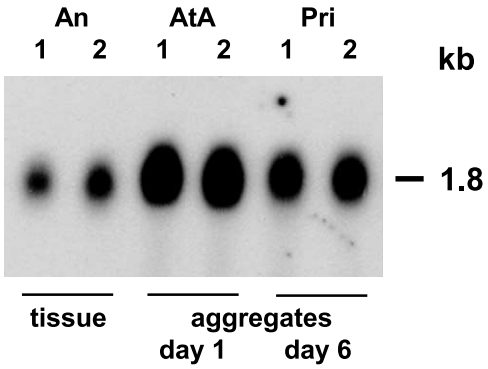

Fig. 4. Northern blot analysis of *Sd-Fz*. RNA from two sponges from the aquarium (An), as well as from adhered 1 day old aggregates (AtA) and 6 days old primmorphs (Pri) was analyzed with a *Sd-Fz* DNA probe. The two different specimens are marked as 1 and 2 for each point analyzed.

the Frizzled family, will contribute interesting data about the origin and the ancestral functions of the Wnt pathway.

Based on recent data that demonstrate the expression of *noggin* in the epithelial layer of the sponge [12] and our data which show the expression of *Sd-Fz* in cells of the pinacoderm layer of the canals, the endopinacocytes, and in cells of the mesohyl directly under the pinacoderm, the exopinacocytes, it is proposed that the stem cells in sponges are localized close to or between the surface layers. This would agree with previous reports proposing that the archeocytes which are accumulated in the mesohyl, between the exopinacocytes and endopinacocytes, are totipotent and are the origin of the differentiation lineages to the terminally differentiated pinacocytes and choanocytes [47]. Therefore, future studies must be performed to elucidate the temporal pattern during canal formation by in situ hybridization.

Recent data also suggest that ingression processes occur in larvae of Demospongiae [48], perhaps indicating that in sponges a primordial gastrulation occurs. Considering that (i) in Triploblasts, both in insects and in vertebrates, an early expression of *Frizzled* is seen in the vicinity of the organizer which determines the difference between the dorsal and ventro-lateral embryonic domains [14], and (ii) also in *Hydra* the Wnt signaling molecules are restricted to the head [49], it will be a task for the future to localize the expression of *Sd-Fz* during sponge embryogenesis. We hypothesize that the expression of *Frizzled* is very likely involved in the formation of an axis in at least some sponges, including also *S. domuncula*. Recently, it was already assumed that primordial axis formation exists in Demospongiae as taken from the presence of LIM/homeobox proteins which are differentially expressed in *S. domuncula*, depending on the attachment to the substratum [9].

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