Int. J. Dev. Biol. 53: 1113-1117 (2009)
doi: 10.1387/ijdb.072526mp

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Heterologous expression of DsRed2 in young sponges *(Porifera)*

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ABSTRACT Sponges (Porifera) are currently considered to be the first branch off the Urmetazoa, common ancestors of all multicellular animals or metazoa. Research in the field of the developmental biology of sponges was restricted to morphological observations. Nowadays, research is mainly concentrated on larval development, primarily dealing with tissue formation. Already since 1907, methods for developing functional sponges from stem cells have been at hand. Functional freshwater sponges can be grown from stem cell populations originating from gemmulae. A number of poriferan sequences with high similarity to regulative genes in higher metazoa have already been found. We have now succeeded in heterologously expressing the red fluorescent protein DsRedN1 under the control of the cytomegalovirus promoter in young specimens of the freshwater sponge *Spongilla lacustris*. The protein folded correctly, polymerized and subsequently was detected by fluorescence microscopy. Reporting this expression system, we now consider this appealing system for early meatazoan development to be ready for molecular developmental biology and functional genetics research.

KEY WORDS: *porifera, freshwater sponge, heterologous expression, functional sponge, DsRed2, CMV*

Introduction

Even though the deep, molecular phylogeny of metazoa is discussed as being not fully resolved, sponges (phylum Porifera) are currently accepted to be the phylogenetically oldest, extant metazoa (Müller, 2003, Erpenbeck and Wörheide, 2007). They evolved during the Neoproterozoic, Neoproterozoic-Cambrian period (Xiao *et al.*, 2005) and are grouped into three classes: the Hexactinellida, the Demospongiae and the Calcarea. Despite their phylogenetic position, branching off directly from the Urmetazoa at the base of all multicellular organisms, their morphology and body plan is already elaborated, even though referred to as modular.

Already in 1907, Wilson investigated the sponges' fascinating ability to regenerate their complex morphology from dissociated cells (Wilson, 1907). Their results already predicted the enormous plasticity of poriferan cell types and showed the sponge cells' ability to recreate a complex body structure from a solution of dissociated cells, which is nowadays exploited for modern biotechnological applications (Nickel *et al.*, 2001). In this process the sponge cells undergo de-differentiation and re-differentiation processes. This is also the case for the two asexual forms of proliferation sponges are capable of, besides sexual proliferation: the simpler way is sheer fragmentation, and the more complex one is the building of gemmulae. The mainly rounded gemmula consists of a durable shell of spongin, which is often times enforced with siliceous gemmuloscleres. It is filled with nursing cells and archaeocytes, which are omnipotent stem cells of the sponge (Weissenfels, 1989). The gemmulae are built to overcome adverse conditions like starvation, freezing, drying as well as anoxia and to facilitate dispersal (Reiswig and Miller, 1998). The dormant or quiescent gemmulae hatch under favourable conditions (Ostrom and Simpson, 1978). During hatching the archaeocytes differentiate and find their place in the newly built morphology of the functional young sponge. Even though sponges show this enormous range of interesting cellular as well as organismic capabilities the porifera are still considered as the very basal metazoan life form (Müller *et al.*, 2004b). Hence they are the organisms of choice to investigate the origin and base of morphogenesis, stem cells, and multicellularity in metazoa (Coutinho *et*

Abbreviations used in this paper: CMV, cytomegalovirus; DsRed, red fluorescent protein derived from Discosoma sp.

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Accepted: 14 April 2008. *Published online:* 22 May 2009.

ISSN: Online 1696-3547, Print 0214-6282
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al., 2003; Müller *et al.*, 2003; Müller *et al.*, 2004c; Perovic-Ottstadt *et al.,* 2004; Martindale, 2005; Steele, 2005). Promising results could already be achieved in identifying genes with high similarities to genes from higher metazoa, which are involved in the regulation of morphogenesis and stem cell regulation or even the regulation of apoptosis (Wiens and Müller, 2006).

Poriferan sequence homologues for the following genes are already identified: the transcription factors *Brachyury* and *Tbx2-3-4-5* from the T-box family, which are involved in the formation of mesodermal and endodermal structures, as well as presumably in the formation of the first body axis (Adell *et al.*, 2003a, Müller *et al.*, 2004b). Five sequences homologous to *Fox* Genes from the *winged-helix/forkhead* family, also involved in higher metazoan organogenesis (Adell and Müller, 2004). The homeodomaine genes *Pax-2/5/8*, from the paired class, and further three genes from the antennapedia and the lim/homeobox class which are up regulated during poriferan canal formation (Hoshiyama *et al.*, 1998, Wiens *et al.*, 2003). A sequence homologue to *Iroquois* genes was also demonstrated to be highly expressed during canal formation (Perovic *et al.*, 2003). The membrane receptor Frizzled is part of the Wnt signal cascade and responsible for tissue polarity. Sequence homologues to Frizzled are shown to be up regulated in poriferan cell re-aggregation (Adell *et al.*, 2003b).

In addition to the growing knowledge on genes presumably involved in morphogenesis, apoptosis and stem cell systems, another prerequisite for functional genetics is already given: for species from the suborder Spongillina Manconi & Pronzato, 2002 comfortable culturing, hatching and regulation procedures are at hand. The sponges can be cultured in successive generations as well as clonal cultures, and sexual proliferation is possible. Even axenic culture techniques are developed (Rozenfeld and Curtis, 1980). A number of chemical and physical possibilities to direct or alter morphogenesis in freshwater sponges is already well documented (Rasmont, 1974, Benfey and Reiswig, 1982, Imsiecke *et al.*, 1994). Hence sterile, synchronized, even clonal cultures are established and at hand. Moreover the morphology of the freshly developed, young sponge is reproducable. Thus abnormalities in morphogenesis are easily detectable. Adult tissue from these cultures can be dedifferentiated to stem cells and used for further studies. Therefore sponges are already discussed to be good models to investigate the evolution of basic features crucial for multicellularity and common to all metazoa (Coutinho *et al.*, 2003, Müller, 2003, Müller *et al.*, 2004a, Müller *et al.*, 2004b). The one obstacle, which is still keeping researchers from experimentally accessing sponges as a system for early metazoan gene function, has been the inability to apply functional genomics with the heterologous gene expression in sponges. If achieved, transgenic sponges will elucidate the function of ancestral genes in the control of morphogenesis, differentiation, immune self recognition and controlled cell death. Morphogenes will be either suppressed or over expressed to result in morphogenetic changes. Similarly appealing systems for the cnidarian *Hydra* are already established and we now would like to go a step further towards the origin of metazoa (Galliot and Schmidt, 2002; Wittlieb *et al.*, 2006; Khalturin *et al.*, 2007; Siebert *et al.*, 2008). Hence we searched for promoters and marker proteins that function in sponges and are detectable after expression. As a promoter we chose the immediate early promoter of the cytomegalovirus (CMV), which is used for high expression levels, especially in mammalian cell cultures. As a marker protein we chose the red fluorescence protein DsRed2, derived from a red fluorescent protein of the coral *Discosoma* sp. Its excitation and emission maxima are way further red than the ones from green fluorescence protein (GFP) derivatives, suggesting DsRed2 as a second marker in co detection experiments with GFP. DsRed2 is also very convenient, because in general the level of background autofluorescence detectable is significantly lower at longer wavelengths. DsRed2 under the control of the CMV promoter is commercially available with the vector pDsRedN1. We here report the first heterologous expression of the marker protein DsRed2 under the control of the CMV promoter in young specimens of the freshwater sponge species *Spongilla lacustris* Linnaeus 1758.

Results and Discussion

Fifteen gemmulae were each placed between two adjacent cover slips on glass object carriers and allowed to hatch (Fig. 1A). After hatching the gross morphology of the young sponges was observed and found to be uniform throughout the complete

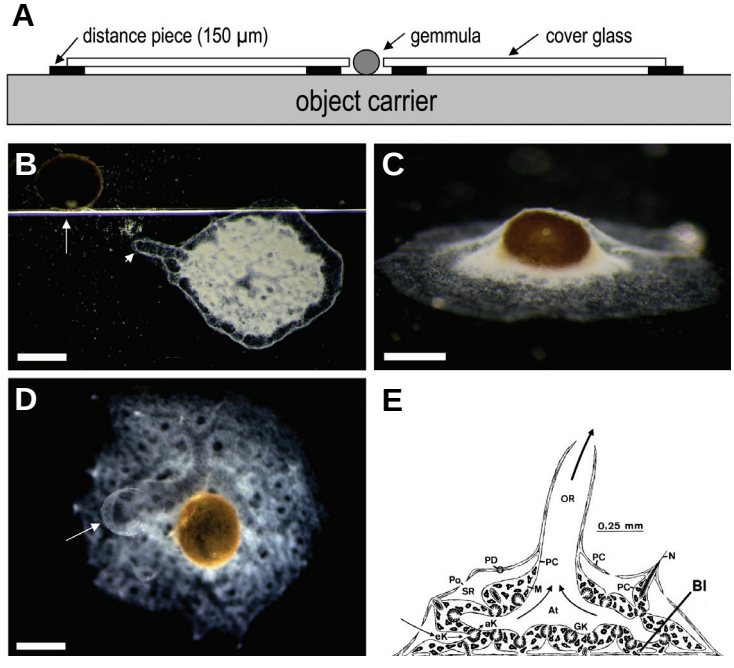

Fig. 1. Gross morphologies of young, functional sponges. (A) *Undercover culture. Setup for two-dimensional growth after hatching.* **(B)** *Typical morphology of a young sponge (top view) grown in the setup shown in (A). The arrow indicates the remaining gemmule. The young sponge grew under a cover glass. The arrowhead indicates the singular oscular tube.* **(C)** *Young sponge after hatching but before the erection of the oscular tube. In this state particle bombardment was performed.* **(D)** *Young sponge after erection of the oscular tube indicated by the arrow (top view).* **(E)** *Schematic drawing of the reproducible morphology of a young sponge (median cut) after (Weissenfels, 1989). eK, incoming canal; GK, choanocyte chamber; aK, outgoing canal; At, atrium; M, mesohyl; SR, subpinacodermal cavity; Po, ostium; PD, pinacoderm; PC, pinacodermal cell; N, spicula; OR, oscular tube; BI, basopinacodermal layer; arrows indicate the water current. Bars, 1 mm.*

sample. All specimens grew two-dimensionally between the cover slip and the object carrier, showed a rounded outline and one oscular tube (Fig. 1B). This reproducibility of the young sponges' gross morphology would clearly make it easy to observe aberrations in the formation of e.g. the first body axis which runs along the oscular tube and is apparently not necessarily depending on gravity.

For the transfection experiments, gemmulae were allowed to hatch on cover glasses. Directly after hatching and before the erection of the oscular tube (Fig. 1C), the specimens underwent particle bombardment to transfect the specimen with the PDsRed2-N1 vector. All fifteen specimens showed the same gross morphology afterwards: the remains of the gemmula were used for scaffolding, the outline was round and one oscular tube was erected perpendicularly to the cover glass (Fig. 1 D,E). Obviously neither the transfection, nor the particle bombardment resulted in aberrant gross morphologies.

We observed living, functional, young sponge specimens, 48 h after transfection. They showed the gross morphology depicted in Fig. 1D and already started to pump water through their canal system (Pfannkuchen *et al.,* 2009). To check for background or autofluorescence, hence the specificity of the applied filter set, we observed untreated specimens and specimens treated with unloaded particles (Fig. 2 G-I). Five untreated specimens showed no cellular fluorescence. Five specimens which were treated with unloaded particle bombardment showed no cellular fluorescence as well. In all five specimens treated with loaded particle bombardment we could find bright cellular fluorescence within the basal cell layer of fully developed young sponges (Fig. 2). We did not enumerate the total number of transfected cells or the transfection rate, because we only observed the basopinacodermal layer. But approximately twenty transfected cells could be found in 1 mm^2 of basopinacoderm. In most cases two or three fluorescent cells could be detected close to each other like shown in Fig. 2 C-F. The fluorescent signal was localized in the cytoplasm but showed a somewhat uneven distribution indicating accumulation of the fluorescent molecules. The outlines of the overlapping cells, the cytoplasmatic fluorescence as well as their nuclei are recognisable in the same focal layer (Fig. 2). The nuclei showed no fluorescence.

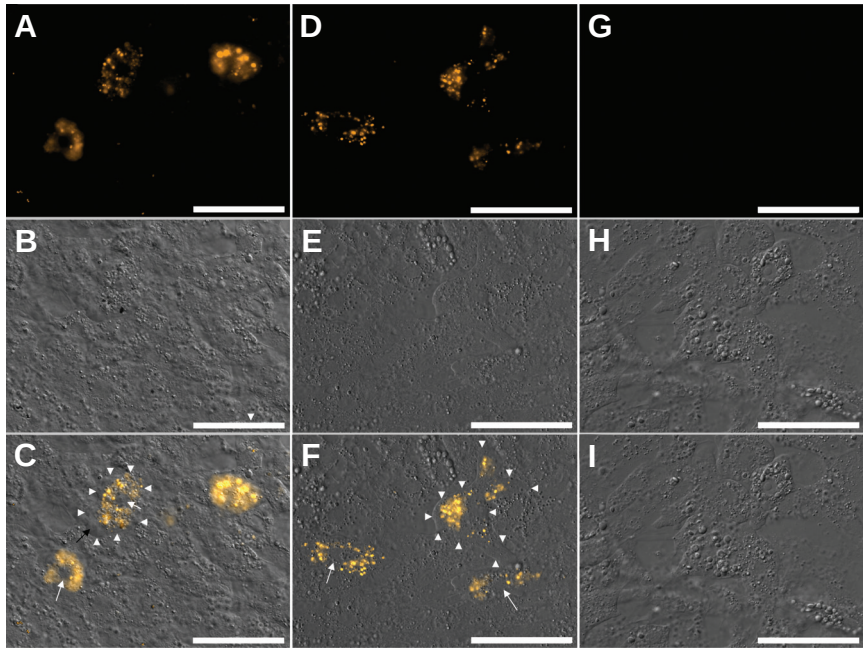

Fig. 2. Cellular fluorescence of DsRed2 in confluent basopinacodermal cells of a living, fully developed young sponge, 48 and 60 h after hatching and subsequent transfection. *(A-C) 48 h after transfection with DsRed2.* **(A)** *Specific fluorescent signal of DsRed2 in three cells.* **(B)** *Differential interference contrast (DIC) image of the corresponding area.* **(C)** *Overlay of the fluorescence and the DIC signal. (D-F) 60 h after transfection with DsRed2.* **(D)** *DsRed2 fluorescence in three cells is appears more restricted to vacuoles.* **(E)** *Corresponding DIC signal and* **(F)** *overlay of both signals. (G-I) Negative control 48 h after bombardment with unloaded particles.* **(G)** *No signal of DsRed2 fluorescence.* **(H)** *Corresponding DIC signal and* **(I)** *overlay of both signals. Cellular outlines are indicated by white arrowheads; nuclei are indicated by white arrows; the black arrow indicates an area of hyaloplasma devoid of fluorescence. Bars, 50 μm.*

Thus, we conclude that after transfection of hatching tissue of *Spongilla lacustris* with the vector PDsRed2-N1 via particle bombardment, the young sponges developed normally and contained transfected cells. These cells expressed high levels of DsRed2 under the control of the CMV promoter. Due to the observation of groups of fluorescent cells, one can hypothesize that the fluorescence is inherited by the daughter cells after cell division. We so far cannot prove this, but rather assume a transient transfection, which would be diluted in daughter cells.

Apparently the fluorescent molecule folded correctly and presumably also polymerized (Baird *et al.*, 2000, Garcia-Parajo *et al.*, 2001, Sacchetti *et al.*, 2002). This shows that PDsRed2-N1 is a suitable vector to transport DNA into sponge cells, and express the transferred sequence. The CMV promoter yielded sufficiently high expression of the marker protein to be detected with fluorescence microscopy. In the described experiments, the young sponges grew on cover slips, which allowed visual inspection of the living specimens' basopinacodermal layer from underneath. Presumably those cells building the attachment site and later the basal layer of the sponge after hatching, have the highest probability to be hit by gold particles, especially as the particles are stopped by the cover glass. Our detection method, using inverse microscopy, allowed very good observations of the basopinacodermal layer. But with this method we did not look for transfected cells further than 300 μm away from the basopinacoderm. Hence, transfected cells which moved away from the basal layer into the sponge body were not observed.

Future experiments will have the DsRed2 sequence replaced by, or coexpressed with, genes of interest (i.e. morphogenes). Now that we proved heterologous expression in functional sponges to be possible, we consider this organism as ready for functional genetics. In addition to looking for sequence similarities, this will allow the investigation of functional homologies of morphogenes and others in the most basal extant metazoa. Five years ago possible constructs for heterologous expression in *Hydra vulgaris* were reported'(Boettger *et al.*, 2002, Miljkovic *et al.*, 2002). We

herewith finally reached the base of the metazoan phylogeny with a functional expression system in freshwater sponges.

Materials and Methods

Sponge material and gemmulae were collected in Lake Constance, Germany by SCUBA diving following the CMAS rules for scientific diving. The gemmulae were directly cooled at 4°C and kept at 4°C for three months.

For hatching single gemmulae were transferred to cover glasses or sandwich constructs in sterile filtered freshwater resembling Rasmonts M-Medium (Rasmont, 1961). After four days at room temperature the gemmulae started to hatch.

For the examination of two-dimensional growth between cover glass and object carrier single gemmulae were positioned inbetween two adjacent cover glasses on an object carrier (Fig. 1A) and were also transferred to M-Medium at room temperature for hatching.

For the particle bombardment single gemmulae were put on cover glasses in M-Medium at room temperature for hatching. One day after hatching, particle bombardment was performed in a Bio-Rad Biolistic Particle Delivery System (PDS-1000/He) (Bio-Rad, Hercules, USA). The distance for the bombardment was 2.5 cm. We used 450 psi rupture disks from Bio-Rad and according macro carriers from Bio-Rad. One µm gold carriers were covered with PDsRed2-N1vector DNA (Clontech Laboratories inc., Mountain View, USA) in a 0.16 mg/ml plasmid solution in water. Subsequently 17 mM Spermidin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and 1.7 M $CaCl_2$ (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was added. After washing the gold particles twice in 98% Ethanol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) they were transferred to the macrocarriers. We applied 0.3 mg gold particles for each bombardment.

Microscopic examination was performed on a Zeiss Axiovert 200 M microscope (Zeiss, Oberkochen, Germany) on living specimens mounted as described on cover glasses. The fluorescent signal of mature DsRed2 molecules (Excitation | Emission Maxima: 558 nm | 583 nm) was detected applying the filterset (BP 550/25 nm | FT 570 nm | BP 605/70 nm) using an Axiocam MR (Zeiss, Oberkochen, Germany). Exposure time was 58 ms. The same exposure time with the same gamma correction values resulted in no signal whatsoever in the untreated specimens as well as in the control specimens after bombardment with unloaded (no vector) particles. Control specimens were tested together with the transfected specimens at 48h after transfection.

Acknowledgements

We are thankful to Reiner Eckert, for help with the PDsRed2-N1 Vector and to Björn Krenz for help with the particle bombardment. This work was supported by the research project BIOTECmarin (03F0414D), funded by the Federal Ministry of Education and Research (BMBF) and the Universität Stuttgart, Germany.

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