Importance of the basement membrane protein SPARC for viability and fertility in *Caenorhabditis elegans*

Michael C. Fitzgerald and Jean E. Schwarzbauer

The basement membrane is a specialized extracellular matrix located at epithelial–mesenchymal boundaries that supports cell adhesion, migration, and proliferation; it is highly conserved between invertebrates and vertebrates [1,2]. One of its component proteins, SPARC (osteonectin/BM-40), binds calcium and collagens, and can modulate cell–matrix interactions, so altering cell shape, growth, and differentiation [3–5]. The tissue distribution of a secreted fusion protein containing SPARC and green fluorescent protein (GFP) was analyzed in *Caenorhabditis elegans.* The protein localized to most basement membranes along body wall and sex muscles, and was also deposited around the pharynx and the gonad, in the spermatheca and at the distal tip cells. The contributions of SPARC to *C. elegans* development were determined using RNA interference, which accurately phenocopies loss-of-function defects [6–8]. A reduction in the amount of SPARC protein resulted in embryonic or larval lethality in a significant proportion of progeny. Those that survived developed a 'clear' phenotype characterized by a lack of gut granules, which made the animals appear transparent, plus small size, and sterility or reduced fecundity. No significant morphological abnormalities were observed, indicating that SPARC plays a regulatory rather than structural role in modulating cell–matrix interactions during normal development and reproduction.

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Received: **14 September 1998**
Revised: **26 October 1998**
Accepted: **26 October 1998**

Published: **9 November 1998**

Current Biology 1998, 8:1285–1288
http://biomednet.com/elecref/0960982200801285

Results and discussion

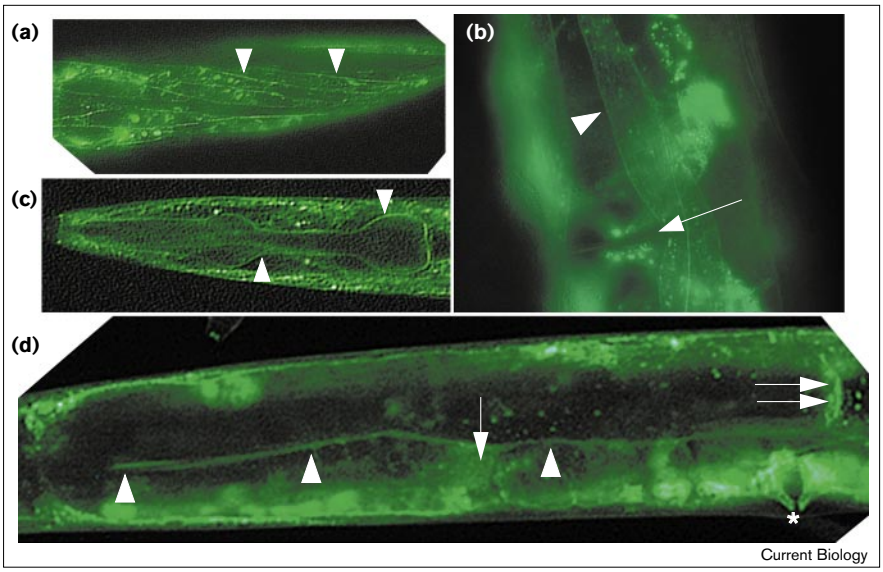
The nematode gene *ost-1*, which encodes SPARC, is expressed at high levels in the body wall and sex muscle cells, as shown using an *ost-1::lacZ* transcriptional fusion gene [3]. To visualize SPARC in whole nematodes, a SPARC–GFP fusion protein, consisting of the signal sequence and first five amino acids of mature SPARC plus green fluorescent protein (GFP) followed by the entire SPARC protein, was expressed in transgenic animals.

Transgenic lines were prepared by microinjection of the *ost-1::GFP* fusion gene either with a *rol-6* dominant marker into N2 wild-type nematodes, or with an *unc-119(+)* wild-type gene into *unc-119* mutants to yield transgenic wild-type revertants. The two types of transgenics gave similar results. An anti-SPARC polyclonal antibody detected the full-length 27 kDa SPARC protein and a band of approximately 54 kDa — appropriately sized for the SPARC–GFP fusion protein — in immunoblots of transgenic lysates (data not shown).

Fluorescence microscopy of transgenic animals showed that SPARC–GFP was localized diffusely along body muscle cells (Figure 1a,b) but did not demonstrate the regular dotted pattern associated with localization to dense bodies or M lines [9]. Fluorescence was limited to areas immediately adjacent to body wall muscle and was concentrated at the boundaries between muscle cells (Figure 1a). Similar distribution patterns were previously reported for type IV collagen and perlecan [10,11]. SPARC–GFP also surrounded the sex muscles, which terminate at the vulva (Figure 1b); it was also detected in embryos and larvae of transgenic animals (data not shown). Although, previously, expression of an *ost-1::lacZ* reporter gene was not detected in pharyngeal muscles [3], in this study SPARC–GFP was localized around the pharynx (Figure 1c); this result suggests that SPARC behaves similarly to type IV collagen, which is expressed by body wall muscle cells but subsequently becomes localized to pharyngeal muscle basement membranes [11]. As SPARC is a matrix-associated protein with collagen-binding activity [5], it seems likely that its localization may be determined by the distribution of integral matrix components such as type IV collagen.

In addition to muscle deposition, SPARC–GFP outlined the entire gonad and was particularly prevalent at the spermatheca and the distal tip cell (Figure 1d). To distinguish whether SPARC is indeed expressed in the gonad or is deposited there after secretion, RNA isolated from dissected body wall, intestines, and gonads was used to prime reverse-transcription-coupled PCR reactions. SPARC mRNA was clearly present in the body wall and gonad tissues but not in the intestine (data not shown). Thus, the gonad is another major site of SPARC expression, along with body wall and sex muscles.

Figure 1



SPARC–GFP is localized to muscle, pharynx, and gonad. Fluorescence microscopy shows **(a)** SPARC–GFP deposited along body wall muscles and concentrated at the boundaries between muscle cells (arrowheads). Rotation of the muscle in this *rol-6*-expressing animal highlights the absence of fluorescence in the body wall between muscle quadrants. **(b)** In addition to its localization to body wall muscle (arrowhead), SPARC–GFP is also abundant along the sex muscles, which terminate at the vulva (arrow). **(c)** Obvious SPARC–GFP fluorescence was seen around the pharynx (arrowheads). **(d)** SPARC–GFP was also found outlining the gonad (arrowheads) and more concentrated at the spermatheca (single arrow) and the distal tip cell (double arrows). Bright fluorescence along the outside edges is due to SPARC–GFP in the body wall and sex muscle. The vulva is indicated (*).

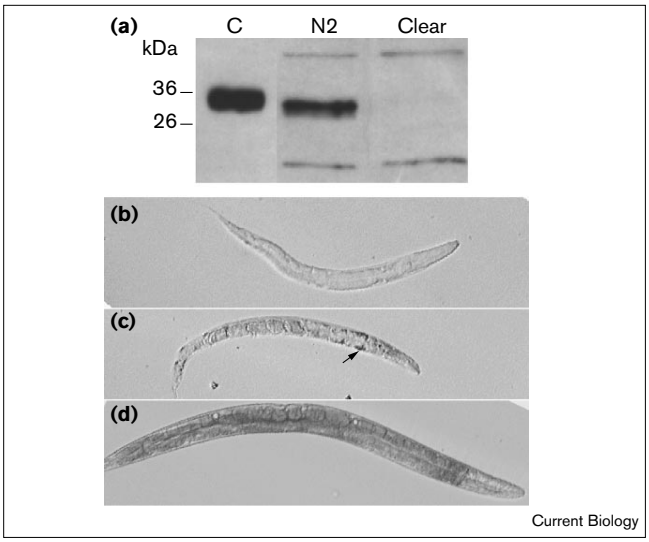
To determine whether a loss of *ost-1* function affected any of these tissues, RNA interference was used to eliminate SPARC in F1 progeny by microinjecting young N2 adult hermaphrodites with double-stranded SPARC RNA [6,7]. Microinjections with fibronectin RNA (as a control) gave wild-type offspring showing no phenotype. In sharp contrast, the affected F1 progeny from hermaphrodites injected with SPARC RNA consistently segregated into two classes: dead embryos or larvae, and clear adult animals. Of 20 injected animals, 6 had 10 or fewer offspring, and the other 14 hermaphrodites produced between 13 and 115 progeny. The proportions of dead versus clear tended to shift with the brood size: in the 6 largest broods (≥ 56 progeny), the majority of affected progeny (52–97%) showed a clear phenotype, whereas, with smaller broods, most of the affected offspring (54–93%) died as embryos or larvae (see Supplementary material published with this paper on the internet). Embryonic lethality occurred at the pre-comma stage, before significant morphogenesis. Dead larvae, which died at around the L1 stage of development, had an apparently normal morphology. As a major site of SPARC expression in early development is the body wall muscle cells, it seems likely that the observed early lethality results, at least in part, from muscle defects that prevent morphogenesis or reduce viability post-hatching, possibly by perturbing muscle cell adhesion or migration [3]. Many of the nematodes that survived past early larval stages developed the clear phenotype, characterized by a lack of gut granules, which was first apparent in larvae at stages L2–L3. This phenotype was due to decreased SPARC expression. A major SPARC band was detected in an immunoblot of a wild-type nematode lysate. In contrast, no SPARC was detected in a lysate from more than three times the number of clear (*ost-1(RNAi)*) nematodes (Figure 2a); the mutant animals therefore do indeed lack SPARC protein.

The *ost-1(RNAi)* L4 larvae completely lack gut granules (Figure 2b) and only a few granules can be seen near the anterior intestine in adults (Figure 2c). The transparent appearance of *ost-1(RNAi)* animals differs from *clr-1* mutants, which look clear because of accumulation of fluid in the pseudocoelom [12]. The genesis of gut granules is poorly understood but they are present in descendents of the E blastomere [13] and appear to play a storage role in the intestine [14]. Perhaps SPARC acts non-cell autonomously to participate in E-cell-specific differentiation.

The *ost-1(RNAi)* adults were about two-thirds the size of wild-type N2 adults, whereas L4 larvae with and without SPARC were equivalent in size (Figure 2b–d). SPARC may affect size indirectly, by causing nutritional deficiencies as a result of the lack of gut granules. Alternatively, the stunted growth could result from a lack of adequate muscle-cell elongation during late larval and adult stages. Clear animals showed no major mobility defects, demonstrating that, in the adult, muscles can function with little or no SPARC protein; paralysis and body-wall deformities resulted from overexpression of SPARC in adult nematodes [3], however, indicating that SPARC can contribute to muscle structure and function.

In addition to changes in size and transparency, almost half (44%) of the *ost-1(RNAi)* hermaphrodites were sterile, while the others had significantly reduced brood sizes — usually less than 10% of the brood size of control-injected hermaphrodites. In 15% of the *ost-1(RNAi)* adults, the embryos developed and hatched in the uterus giving a

Figure 2



Animals injected with *ost-1* RNA lack gut granules and are small. **(a)** Five wild-type N2 nematodes and 16 clear animals were lysed in SDS–PAGE sample buffer containing reducing agent and immunoblotted with an anti-SPARC antiserum along with a control lysate (C) known to contain SPARC (background bands above and below SPARC are contaminating OP50 bacterial proteins). Clear L4 larva **(b)** and adult **(c)** animals are shown in comparison to an adult N2 hermaphrodite **(d)**. Note the transparent appearance of clear animals; a few gut granules can be seen in the adult (arrow). The *ost-1(RNAi)* L4 larvae are a normal size but the adults are small relative to N2 (compare c with d).

'bag-of-worms' phenotype (data not shown). Distal gonad and oocyte morphologies appeared normal, suggesting that the defect(s) occurred at, or following, fertilization. Interestingly, overexpression of SPARC had a similar outcome: oocytes appeared normal but embryos were deformed and nonviable [3]. Given that SPARC can affect cell–matrix interactions, its absence may impinge on the ability of reproductive muscle cells to function effectively. Perhaps inefficient contraction by the myoepithelium of the gonad and/or the sex muscles results in decreased movement of oocytes through the spermatheca and slower expulsion of embryos from the uterus [15]; this model would explain the sterility, reduced fecundity, and bag-of-worms phenotype, even with normal gonad morphology. Changes in cell interactions with surrounding tissues could be directly affected by SPARC or may be mediated by other matrix components regulated by SPARC. For example, the production of metalloproteases [16] and plasminogen activator inhibitor-1 expression [17] are stimulated by SPARC, and a reduction of these types of proteins in SPARC-deficient gonads or muscles could affect tissue function.

Our results demonstrate that SPARC is required for the completion of normal development and affects the function of a variety of tissues. The viability and fertility of SPARC-null mice [18,19] is contrary to our observations with *C. elegans* SPARC loss-of-function mutants. However, SPARC-null mice do develop severe early-onset cataracts and lens rupture, supporting a role for SPARC in proper lens cell differentiation [18,19]. Perhaps one of the at least four other vertebrate SPARC family members functionally compensate for the absence of SPARC in other tissues [20–23]. A search of the *C. elegans* database for homologues of SPARC revealed no matches; *ost-1* appears to be a unique gene in nematodes (our unpublished observations). Thus, further analyses of SPARC function in the nematode could provide insights into its precise role in modulating cell–matrix interactions.

Materials and methods

Maintenance and production of transgenic nematodes

Wild-type N2 and transgenic *C. elegans* and *unc-119* mutants were maintained using standard conditions [24,25]. Healthy hermaphrodites at the late L4 or early adult stage were used for DNA microinjections. The pOST8-GFP plasmid at either 1 or 0.2 μg/ml was co-injected with one of two different marker genes, which were used at 100 μg/ml: pRF4 DNA carrying *rol-6(su1006)* into N2 animals [26] or MM016 DNA with *unc-119*$^{(+)}$ into *unc-119(e2498)* mutants [27]. Transgenic animals showing either Rol or wild-type Unc-119$^{(+)}$ phenotypes (depending on the marker gene) were analyzed for expression of SPARC–GFP by fluorescence microscopy using a Nikon Optiphot-2 microscope. Images were captured using a cooled charge-coupled device (3-CCD) video camera (DEI-750, Optronics Engineering) connected to a Macintosh G3 computer equipped with an LG3 board (Scion Corp.). For differential interference contrast microscopy, a Nikon Diaphot inverted microscope was used with an NEC camera connected to a Macintosh 8600 computer with AV capabilities.

Construction of ost-1::GFP *fusion gene*

A plasmid carrying the entire *ost-1* gene plus 5′ and 3′ flanking sequences was engineered for insertion of a GFP gene in place of the first intron, which falls between sequences encoding the signal sequence and the amino-terminal acidic domain of SPARC [3]. *Xba*I sites were engineered by PCR amplification using primers homologous to the 3′ and 5′ ends of exons A and B, respectively, in combination with upstream and downstream primers homologous to sequences within *ost-1*. Products were digested with *Xba*I and either *Eco*RV (upstream) or *Pst*I (downstream) and cloned into the SPARC gene generating the plasmid pOST8-X1/2. *Xba*I sites were also added to both ends of the GFP (S65T) gene from pPD95.85 (kindly provided by A. Fire) using primers with in-frame *Xba*I sites at the 5′ ends to amplify the gene. Sequences of all PCR products were confirmed. The GFP gene was inserted into the *Xba*I site of pOST8-X1/2 to generate pOST8-GFP, which was then purified by CsCl centrifugation.

SDS–PAGE and immunoblotting

For lysis of defined numbers of animals, nematodes were picked from plates into 10 μl M9 buffer [25]. After pelleting, SDS and DTT were added to a final concentration of 2% and 0.1 M, respectively. Samples were boiled to lyse nematodes and solubilize proteins. Since nematodes are grown on a lawn of OP50 bacteria, individuals picked from culture plates are contaminated with these bacteria. To control for bacterial background bands, OP50 bacterial lysates were prepared in the same way. Lysates were separated by electrophoresis through a 12% polyacrylamide–SDS gel, followed by transfer to nitrocellulose. SPARC was detected using anti-polyclonal antiserum 1051 [5] at 1:400 dilution followed by incubations with horseradish peroxidase-conjugated goat anti-rabbit IgG (Gibco-BRL) and chemiluminescence reagents (Pierce).

RNA interference (RNAi)

Single-stranded RNA was prepared by transcription *in vitro* using a Bluescript plasmid containing the entire 1.1 kb SPARC cDNA beginning 16 bp upstream of the ATG and ending at the polyA addition site [3]. The template was amplified using Bluescribe2 (Stratagene) and M13 primers and reverse-transcribed using Riboprobe reagents and either T3 or T7 polymerase (Promega Biotech). Control fibronectin RNA of approximately the same size was also prepared. Conditions for RNA preparation prior to microinjection were as described by Fire *et al.* [7]. Double-stranded RNA was prepared by incubation of equal amounts of single-stranded RNA in 3×IM buffer [7,25] and was microinjected into the gonads of young adult hermaphrodites. Fertilized eggs were allowed to clear for 4–5 h and progeny collected during the following 22 h were analyzed for phenotypes.

Supplementary material

A table describing the phenotypes of the progeny of adults injected with SPARC RNA is published with this paper on the internet.

Acknowledgements

We thank Frederique Musset-Bilal and Carol S. Ryan for contributions to the early stages of this work, to Andrew Fire (Carnegie Institution of Washington) for providing pPD95.85, and to Myeongwoo Lee for critical reading of the manuscript and helpful discussions. This work was supported by the American Cancer Society.

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Supplementary material

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Current Biology 9 November 1998, **8**:1285–1288

Table 1

Phenotypes of the progeny of adults injected with SPARC RNA.

Number of offspring	Proportion of progeny affected (%)	Proportion of those affected with each phenotype (%)	
		Dead	Clear
13	54	86	14
19	79	93	7
24	83	60	40
27	81	86	14
31	71	68	32
42	62	54	46
43	47	55	45
55	64	63	37
56	52	48	52
62	40	8	92
69	45	32	68
99	28	36	64
114	32	3	97
115	37	44	56

F1 progeny were followed after microinjection of double-stranded SPARC mRNA. The total number of offspring per adult and number that showed each of the phenotypes were counted. The ‘dead’ category includes dead embryos and larvae, numbers of which were approximately equal in each case. The ‘clear’ category refers to surviving offspring that showed the clear phenotype described in the text. Only animals laying more than 10 eggs were included in the table.