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Endo16 is required for gastrulation in the sea urchin *Lytechinus variegatus*

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The *Endo16* gene encodes a large extracellular protein with several functional domains that provide some insight into the role of this protein during embryonic development. We isolated the full-length cDNA sequence from *Lytechinus variegatus* and utilized morpholinos to further investigate the role of Endo16 during embryonic development in this species. Endo16-deficient embryos failed to undergo gastrulation and the blastocoele became filled with dissociated cells after 24 h of incubation. Moreover, there was a delay in endoderm differentiation as assayed by staining with an antibody that recognizes Endo1. The differentiation of other cell types including oral ectoderm, primary mesenchymal cells (PMC) and secondary mesenchymal cells (SMC) appeared to be normal, although the patterns of protein expression did not resemble control embryos due to the gross morphological abnormalities elicited by the LvEndo16 morpholino. Microinjection of full-length *EGFP* mRNA with the LvEndo16 morpholino-targeted sequence confirmed that this phenotype can be attributed specifically to the loss of Endo16 protein. Taken together, our data suggest that Endo16 may be required for the cell–extracellular matrix (ECM) interactions that are required for endoderm differentiation in the sea urchin embryo.

Key words: antisense, *Endo16*, endoderm, morpholino, sea urchin.

Introduction

The transcriptional regulation of *Endo16* has been characterized to a greater extent than nearly any other gene (Yuh *et al*. 1994; Yuh & Davidson 1996; Yuh *et al*. 1996, 1998, 2001; Romano & Wray 2003), yet the function of Endo16 protein during sea urchin development is still unclear. The *Endo16* gene encodes a large protein that is secreted into the extracellular matrix at early stages of embryonic development (Soltysik-Espanola *et al*. 1994). *Endo16* was originally isolated from *Strongylocentrotus purpuratus* by screening a gastrula stage cDNA library (Nocente-McGrath *et al*. 1989). The gene was designated *Endo16* based on its endoderm-specific pattern of expression (Nocente-McGrath *et al*. 1989). *Endo16* is initially expressed in the vegetal plate of the hatched blastula (Nocente-McGrath *et al*. 1989; Ransick *et al*. 1993). *Endo16* expression is downregulated in primary mesenchymal cells (PMC) as they migrate away from the center of the vegetal plate to form the larval skeleton. During gastrulation, *Endo16* is expressed throughout the invaginating archenteron. *Endo16* expression is downregulated in secondary mesenchymal cells (SMC) as they migrate away from the anterior tip of the archenteron to form pigment cells. At the end of gastrulation, *Endo16* expression is downregulated in the anterior 1/3 of the archenteron, which corresponds to the prospective foregut (pharynx). Later, *Endo16* expression is also downregulated in the posterior 1/3 of the archenteron, which corresponds to the prospective hindgut (intestine). *Endo16* expression is maintained in the midgut (stomach) of the pluteus larva. In a different species of sea urchin, *Lytechinus variegatus*, *Endo16* expression is maintained in both the midgut and hindgut of the pluteus larva (Romano & Wray 2003).

Transient expression assays demonstrated that 2.2 kb of sequence immediately upstream of the transcriptional start site is sufficient to drive this pattern of *Endo16* expression in *S. purpuratus* (Yuh *et al*. 1994). Recently, it was shown that 2.2 kb of upstream sequence also is sufficient to drive *Endo16* expression in *L. variegatus* (Romano & Wray 2003). Interestingly, only the most proximal region of the *Endo16*

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promoter, module A, is conserved between these two species. Reciprocal cross-species transient expression assays revealed that there has also been evolutionary divergence in the set of transcription factors that interact with the *Endo16* promoter (Romano & Wray 2003). These observations suggest that there has been stabilizing selection on the transcriptional output of the *Endo16* promoter.

In the present study, we have used morpholino-substituted antisense oligonucleotides ('morpholinos') to gain insight into the function of Endo16 during sea urchin development. Morpholinos are designed to 'knockdown' protein synthesis by hybridizing with the targeted mRNA at or near the site of translation initiation (Summerton 1999). In contrast to other gene targeting strategies, morpholinos display little toxicity and have been shown to inhibit translation by nearly 100% in several systems, including the sea urchin (reviewed by Ekker & Larson 2001; Angerer & Angerer 2004). Here, we demonstrate using morpholinos that Endo16 is essential for gastrulation in *L. variegatus*.

Materials and methods

Preparation of cultures

L. vareigatus adults (collected near Beaufort, NC, USA) were maintained in an aquarium at room temperature. Gametes were obtained by injecting adults with 0.55 M KCl. Following fertilization, the eggs were cultured at room temperature in artificial seawater until the desired stages.

Isolation of full-length LvEndo16 cDNA sequence

RNA was isolated from gastrula-stage embryos using RNA STAT-60 (Tel.Test 'B', Friendswood, TX, USA) and treated with DNase (Gibco BRL, Gaithersburg, MD, USA). Reverse transcription (RT) was performed according to the instructions provided by the SuperScript Reverse Transcription kit (Gibco BRL). Following the addition of a poly(A) tail, the cDNA was used to perform 5′ and 3′ rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR). Primers were based on a partial cDNA sequence previously reported by Godin *et al.* (1997) (GenBank accession #U89340). PCR products obtained by 5′ and 3′ RACE PCR were gel purified and ligated into pGEM-T vector (Promega, Madison, WI, USA). Plasmid DNA was purified from transformed DH5α cells (Gibco BRL) and sequenced using Big Dye and an ABI Prism 3700 DNA Analyzer (PE Applied Biosystems, Foster City, CA, USA). Sequences were assembled using Sequencher software (Gene Codes, Ann Arbor, MI, USA).

Microinjection of morpholinos

A morpholino was designed based on the 5′ region of the *LvEndo16* cDNA sequence and obtained from GeneTools, LLC (Philomath, OR, USA). We tested an unmodified morpholino as well as a morpholino with 3′-conjugated lissamine, a modification that allowed us to distinguish Endo16-deficient embryos by fluorescence microscopy. The sequence of the LvEndo16 morpholino was: 5′ CCAAGCAAAAAAGTTCCTTCCGCAT and corresponded to +1 to +25 of the *LvEndo16* transcript. It was not possible to use any other sequence surrounding the translational start site due to internal complementarity. The sequence of the standard control morpholino that was supplied by GeneTools, LLC was: 5′CCTCTTACCTCAGTTACAATTTATA. We tested a range of morpholino concentrations including 100 μM, 200 μM, 300 μM and 400 μM to determine the most effective concentration for producing an abnormal phenotype with little or no toxicity. The morpholino was diluted in water to the appropriate concentration with 30% glycerol and spun at 13 000 r.p.m. for 5 min through a Millex-GV filter unit (Millipore, Bedford, MA, USA).

Eggs were dejellied by incubating in artificial seawater, pH 5.0 for 1.5 min. The eggs were then transferred to plastic Petri dishes coated with 1% protamine sulfate. The total number of eggs was recorded for each Petri dish. Eggs were microinjected using a PLI-100 Picospritzer (Medical Systems, Greenvale, NY, USA) under an Axiovert S100 inverted microscope (Zeiss, Jena, Germany). Following microinjection, the *L. variegatus* eggs were fertilized and cultured at room temperature. After 24 h, embryos and larvae were observed under a Axioskop MOT II microscope (Zeiss) equipped for fluorescence microscopy. We counted the number of normal and abnormal embryos that exhibited red fluorescence produced by the 3′ lissamine, as well as the total number of surviving embryos in each Petri dish in order to determine the lethality rate of injected eggs at a particular morpholino concentration. Images of fluorescent embryos were captured using a digital camera (Hamamatsu, Model #C4742-95-12R, 'Orca'; Hamamatsu City, Japan) and analyzed using Openlab 2.2.4 (Improvision, Lexington, MA, USA). At 24, 48 and 72 h postfertilization, images of normal and abnormal embryos were recorded using a Spot camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Microinjection of LvEndo16-M-GFP *construct*

The full-length *EGFP* gene was amplified by PCR from pEGFP-1 vector (Clontech, Palo Alto, CA, USA) using primers with restriction sites on their 5′ ends in order to facilitate directional cloning. The 5′ primer

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also contained the morpholino-targeted sequence, ATGCGGAAGGAACTTTTTTGCTTGGTC, immediately upstream of the translational start site for EGFP. The sequence of the 5′ primer was: 5′ TAGAATTCATGCG-GAAGGAACTTTTTTGCTTGGTCGGAATGGTGAGCAA-GGGCGAGGAGCTGTTC, and that of the 3′ primer was: 5′ TACTCGAGTTACTTGTACAGCTCGTCCATGCCGAG. The primers were designed such that the morpholino-targeted sequence would be in frame with EGFP resulting in a fusion protein. The PCR product was digested with *Eco*RI and Xho1 and ligated into pCS2+ vector. The ligation product was cloned and sequenced as described above. The *LvEndo16-M*-GFP construct was linearized with Not1. We then performed *in vitro* transcription using SP6 RNA polymerase according to the instructions provided by the mMessage mMachine kit (Ambion, Austin, TX, USA). Eggs were microinjected, as described above, with RNA (~2 μg/μL) diluted at 1 : 50 along with 200 μM LvEndo16 or standard control morpholino. Images of fluorescent embryos were captured as previously described.

Antibody staining

Injected embryos were collected after 24 h or 72 h and fixed with 4% formaldehyde for 1 h at room temperature. Fixed embryos were dehydrated through 70% ethanol and stored at 4°C. Embryos were rehydrated through 30% ethanol and rinsed three times in phosphate-buffered saline (PBS) for 10 min at room temperature. Embryos were permeabilized with ice cold methanol for 90 s and rinsed four times with PBS for 10 min at room temperature. Embryos were blocked with 4% normal goat serum in PBS for 1 h at room temperature prior to incubation with primary antibody. In this study, we used four monoclonal antibodies as cell-type specific markers: Endo1, EctoV, 1d5, and SMC-1. The primary antibody was diluted to the appropriate concentration (Endo1 and EctoV, undiluted; 1d5, 1 : 4; SMC-1, 1 : 50) with 4% normal goat serum in PBS. Embryos were incubated with primary antibody overnight at 4°C (Endo1, EctoV, and SMC-1) or for 2 h at room temperature (1d5). Embryos were rinsed four times with PBS for 10 min at room temperature. A fluorescein-conjugated goat antimouse antibody (Molecular Probes, Invitrogen, Carlsbad, CA, USA) was diluted to 1 : 500 with 4% normal goat serum in PBS. Embryos were incubated with secondary antibody for 2 h at room temperature and rinsed as described above. Finally, embryos were resuspended in PBS with 70% glycerol and 0.5% n-propyl gallate (Sigma, St. Louis, MO, USA) and stored in the dark at 4°C. Images of fluorescent embryos were captured as previously described. Endo1, EctoV and 1d5 antibodies were kindly provided by Dave McClay (Duke University) and the SMC-1 antibody was kindly provided by Chuck Ettensohn (Carnegie Mellon University).

Results

Isolation of full-length LvEndo16 *cDNA sequence*

Reverse transcription was performed using RNA isolated from gastrula-stage embryos. Full-length *LvEndo16* cDNA sequence was obtained by 5′ and 3′ RACE PCR using primers based on a partial *LvEndo16* cDNA sequence previously reported by Godin *et al.* (1997). The full-length *LvEndo16* mRNA sequence is 4565 bp in length and encodes a predicted protein that consists of 1485 amino acids (Fig. 1) (GenBank accession #DQ387433). In contrast, the full-length *SpEndo16* mRNA sequence is 4692 bp in length and encodes a predicted protein that consists of 1560 amino acids. Therefore, the LvEndo16 protein appears to be slightly shorter than the SpEndo16 protein. LvEndo16 and SpEndo16 are overall 63.4% similar at the amino acid level. As in *S. purpuratus*, there is a signal sequence at the N terminus of LvEndo16, consistent with data indicating that Endo16 is a secreted protein (Soltysik-Espanola *et al.* 1994). The predicted amino acid sequence indicates that the LvEndo16 protein has several additional features in common with its homologue in *S. purpuratus*. For example, there are numerous cysteine residues near the N terminus that are arranged in a pattern similar to that of proteins belonging to the serum albumin family, where the disulfide bonds have been shown to be responsible for ligand binding (Kragh-Hansen 1990). In addition, there is an RGD motif indicating that Endo16 may associate with the cell surface via an integrin-like receptor (Ruoslahti 1996). Finally, there are two regions of clustered repeats, which have been shown to bind to calcium, near the C terminus (Soltysik-Espanola *et al.* 1994). However, the C terminus, which is very acidic, is considerably less conserved between the two species.

In order to gain more insight into the structure and potential function of the Endo16 protein, we utilized the Conserved Domains Database available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Endo16 contains an albumin domain that is typical of proteins belonging to the albuminoid superfamily (Fig. 1). Proteins belonging to the albuminoid superfamily appear to be derived from a duplication of the gene encoding vitamin D binding protein and many of them have been shown to bind to fatty acids and other macromolecules (Yang *et al.* 1985). In addition, Endo16 contains a Reeler domain of unknown function that is also found at the

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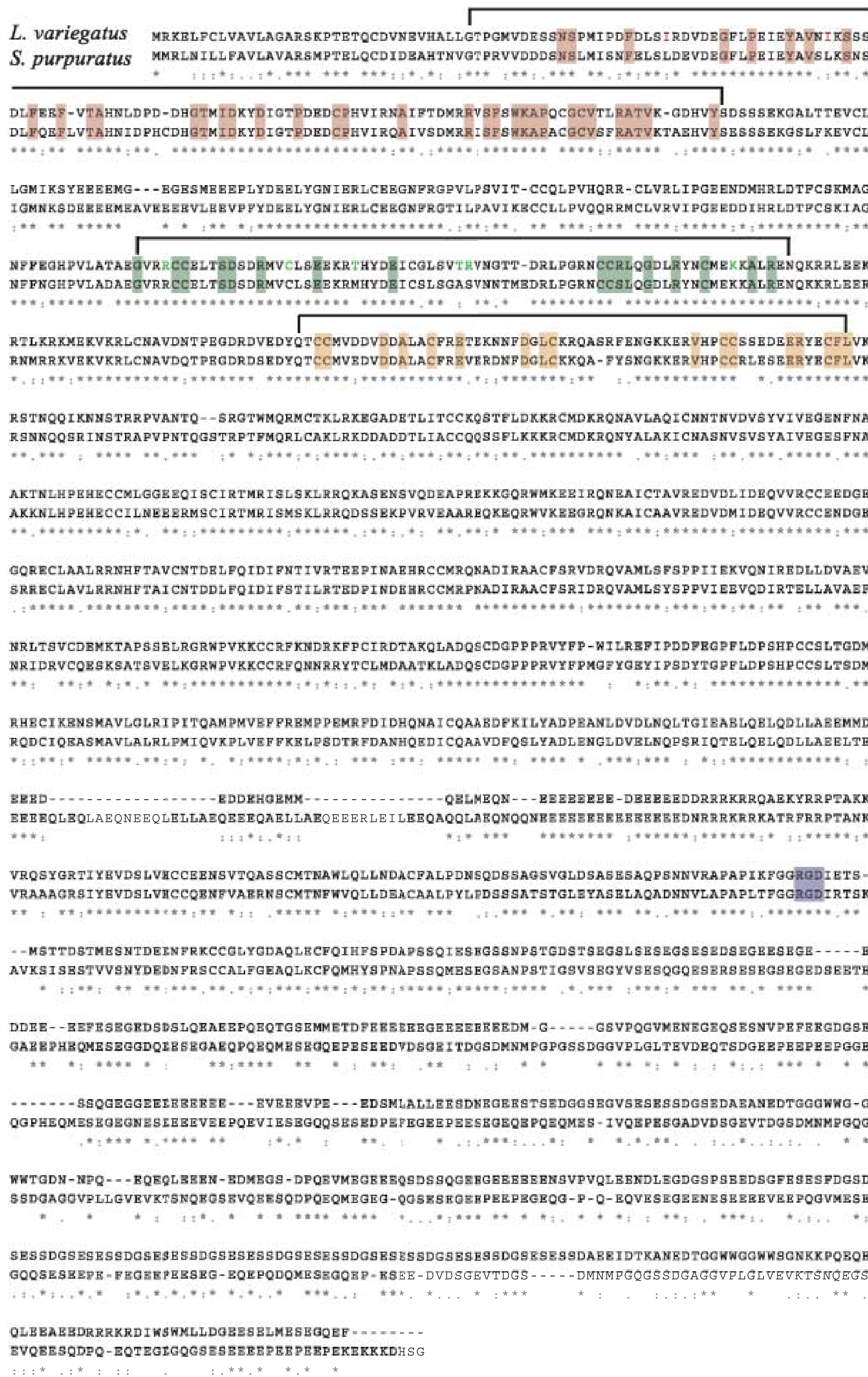

Fig. 1. Comparison of Endo16 protein in *L. variegatus* and *S. purpuratus*. An alignment of the predicted amino acid sequences for LvEndo16 and SpEndo16 was performed using Clustal-W. Amino acids highlighted with a red box are those conserved between Endo16 and the Reeler domain, while those highlighted with an orange box are those conserved between Endo16 and the Albumin domain, according to the Conserved Domain Database available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The region containing amino acids highlighted with a green box exhibits significant sequence similarity to ECM1. The RGD sequence is denoted with a blue box.

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N terminus of several proteins including Reelin, which plays a role in vertebrate nervous system development (Fig. 1) (Fatemi 2005). Finally, a region of Endo16 showed significant sequence similarity to ECM1, a large extracellular matrix protein of unknown function that is associated with connective tissue in vertebrates (Fig. 1) (Bhalerao *et al.* 1995). A BLAST search confirmed that Endo16 is similar to ECM1 as well as several hypothetical proteins of unknown function.

Disruption of Endo16 function using morpholinos

To determine the function of Endo16 during sea urchin development, we designed a morpholino based on the 5′ region of the isolated *LvEndo16* cDNA sequence. We used a 25 bp morpholino with a 3′-conjugated lissamine dye in order to identify Endo16-deficient embryos by fluorescence microscopy. The targeted sequence corresponded to +1 to +25 of the coding region; a BLAST search revealed that the morpholino did not match any other sequence in the sea urchin genome database. We also tested a standard control morpholino available from GeneTools, LLC. Injection of the standard control morpholino had no discernible effect on embryonic development as compared to uninjected embryos (Fig. 2).

Prior to hatching, the LvEndo16 morpholino did not appear to have any effect on development, although

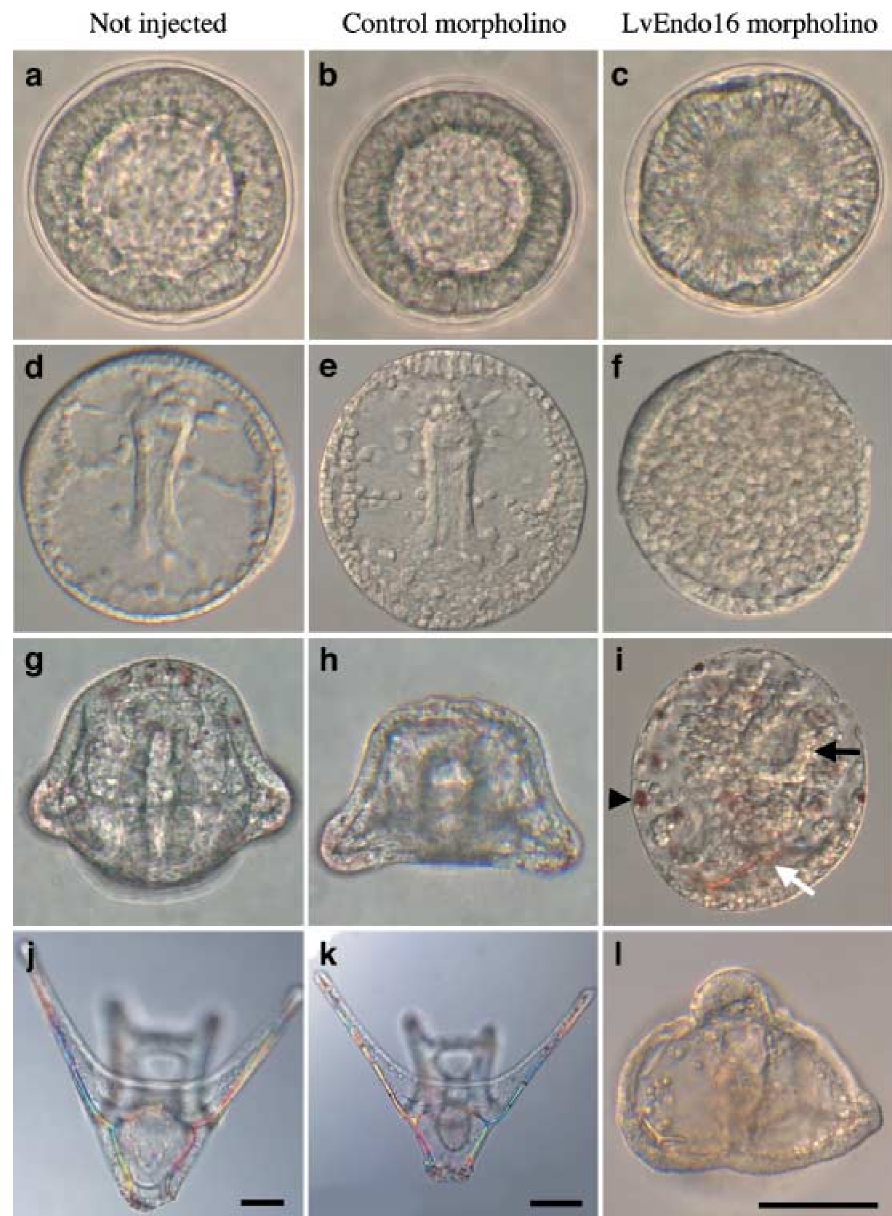

Fig. 2. Effect of morpholinos on the development of *L. variegatus*. Embryos were observed prior to hatching (a, b and c) as well as after ~24 h (d, e and f), 48 h (g, h and i), and 72 h (j, k and l). Uninjected embryos as well as embryos injected with the standard control morpholino developed into pluteus larvae with a complete gut (j, k). In contrast, embryos injected with the LvEndo16 morpholino developed abnormally. After ~24 h, LvEndo16-deficient embryos had failed to undergo gastrulation (f). After ~48 h, pigment cells (black arrowhead) and skeletal elements (white arrow) were present although disorganized in LvEndo16-deficient embryos (i). In some cases, small archenteron-like structures (black arrow) had also formed. After ~72 h, LvEndo16-deficient embryos displayed gross morphological abnormalities with few surviving beyond this time period (l). Scale bar = 100 μm.

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some of the Endo16-deficient embryos (Fig. 2c) were not as spherical as control embryos (Fig. 2a,b). The phenotype of Endo16-deficient embryos became quite apparent around the time of gastrulation. After ~24 h in culture, uninjected embryos (Fig. 2d) as well as embryos injected with a standard control morpholino (Fig. 2e) had formed an archenteron. In contrast to control embryos (Fig. 2d,e), invagination of the vegetal plate did not occur in embryos injected with the LvEndo16 morpholino (Fig. 2f). However, the blastocoele became filled with dissociated cells of unknown origin (Fig. 2f). By ~48 h, pigment cells and small skeletal elements were apparent within the blastocoele of embryos injected with the LvEndo16 morpholino (Fig. 2i). Although they contained visibly differentiated cell types, Endo16-deficient embryos lacked both the skeletal patterning and ectodermal invasion of pigment cells exhibited by control embryos (Fig. 2g,h). By ~48 h, a small archenteron-like structure was recovered in some of the LvEndo16-deficient embryos, although it rarely fused with the ectoderm to form a complete gut (Fig. 2i). Both uninjected embryos (Fig. 2i) as well as embryos injected with a standard control morpholino (Fig. 2k) developed into four-arm pluetus larvae with a functional gut after ~72 h of incubation. LvEndo16-deficient embryos displayed gross abnormalities and most did not survive beyond this time-point (Fig. 2l).

We tested the effect of morpholinos at concentrations ranging from 100 μM to 400 μM. By counting embryos that exhibited a strong red fluorescent signal (Fig. 3), we found that 200 μM was an optimal morpholino concentration for producing an abnormal phenotype without having a considerable effect on the survival of embryos up to ~72 h in culture (Fig. 4). Only 21% of embryos injected with the LvEndo16 morpholino at a concentration of 100 μM displayed an abnormal phenotype (Fig. 4a). In contrast, 70% of embryos injected with the LvEndo16 morpholino at a concentration of 200 μm exhibited an abnormal phenotype (Fig. 4a); injection of an unmodified morpholino at this concentration produced similar results with approximately 78% of embryos affected by the Endo16 deficiency (data not shown). Higher concentrations resulted in a slight increase in the efficacy of the LvEndo16 morpholino (Fig. 4a). However, it should be noted that embryos injected with morpholinos at concentrations above 300 μM exhibited a considerable decrease in survival (Fig. 4b).

Because a reliable antibody is not available for Endo16 that would allow us to directly monitor protein expression, we performed an additional control for these experiments. We constructed an artificial mRNA that included the full-length *EGFP* mRNA with the LvEndo16 morpholino-targeted sequence inserted into its 5′ UTR. This mRNA, referred to as *LvEndo16-M*-GFP, was injected into *L. variegatus* eggs either alone or in combination with the LvEndo16 or standard control morpholino. Injection of *LvEndo16-M*-GFP into *L. variegatus* eggs in the absence of morpholino produced a strong green fluorescent signal (Fig. 5a). Co-injection of the standard control morpholino also produced green fluorescence due to its inability to hybridize with the artificial mRNA and block translation (Fig. 5b). In contrast, the green fluorescence was eliminated in nearly 100% of the embryos co-injected with the LvEndo16 morpholino (Fig. 5c), and many of the embryos failed to undergo gastrulation as previously described. We believe that these data confirm the specificity of the LvEndo16 morpholino and its effect on the development of *L. variegatus*. Similar controls using green fluorescent protein (GFP) have been performed to confirm the specificity of morpholinos during sea urchin development (e.g. Angerer *et al.* 2001; Howard *et al.* 2001; Duboc *et al.* 2005). This approach was originally taken by Nasevicius *et al.* (2000), who tested the effect of a morpholino directed against vascular endothelial growth factor (VEGF)-A during zebrafish development.

Antibody staining of LvEndo16-deficient embryos

The blastocoele of Endo16-deficient embryos became filled with dissociated cells of unknown identity. We

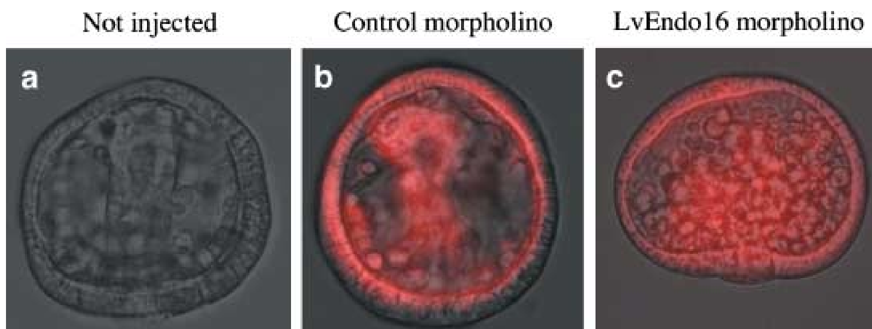

Fig. 3. Use of fluorescence to distinguish embryos injected with morpholinos. Embryos were injected with morpholinos tagged with a 3′ lissamine so that they could be distinguished from uninjected embryos by fluorescence microscopy. Uninjected embryos exhibited no fluorescence (a). In contrast, embryos injected with the standard control morpholino (b) or the LvEndo16 morpholino (c) exhibited a strong red fluorescent signal.

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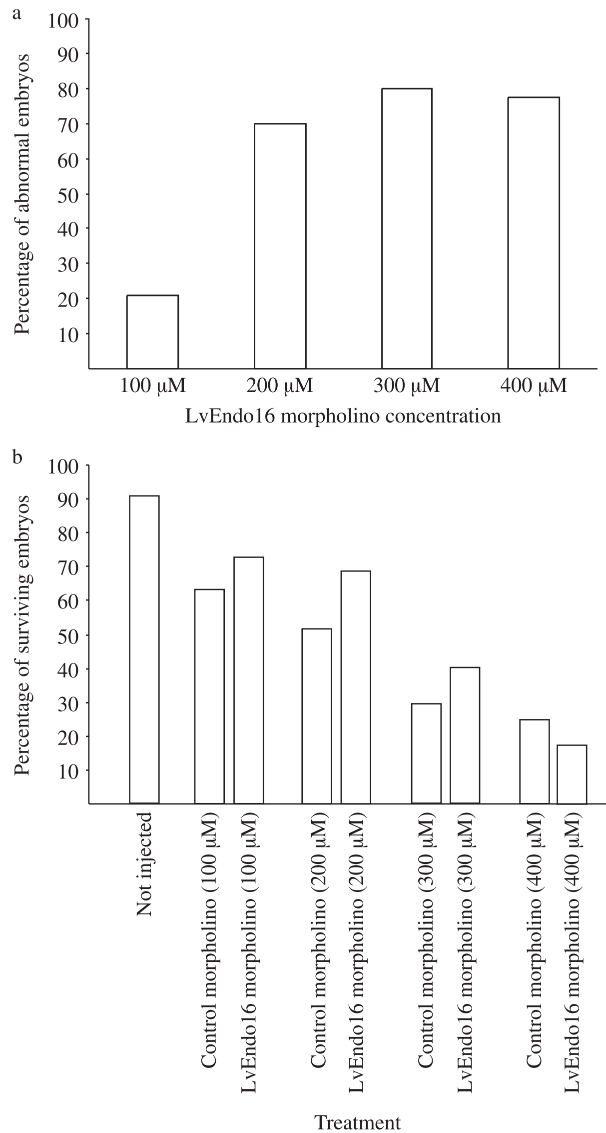

Fig. 4. Effect of morpholino concentration on the survival of *L. variegatus*. (a) Percentage of abnormal embryos injected with different concentrations (100, 200, 300 and 400 µm) of the LvEndo16 morpholino. Only LvEndo16-deficient embryos that exhibited red fluorescence were examined after ~72 h of incubation. (b) Percentage of embryos injected with different concentrations (100, 200, 300 and 400 µm) of the standard control morpholino or the LvEndo16 morpholino that were alive after ~72 h of incubation as compared to uninjected embryos. N > 150 for each treatment.

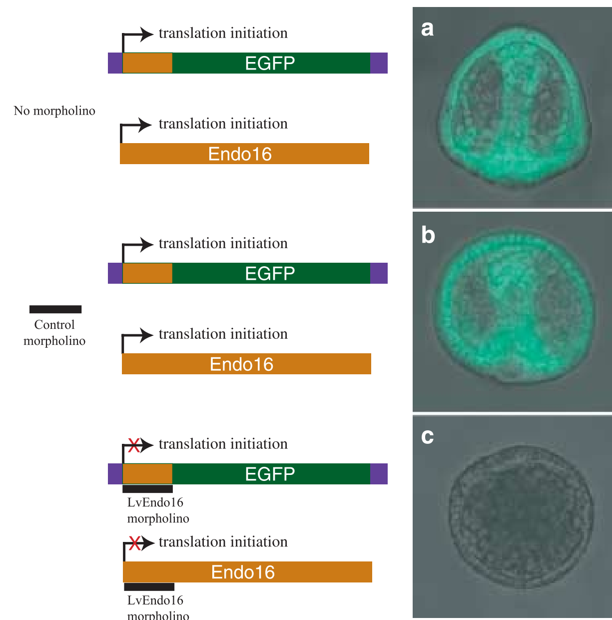

Fig. 5. Microinjection of *LvEndo16-M*-GFP construct to test the specificity of the LvEndo16 morpholino. We prepared an *LvEndo16-M*-GFP construct that encoded a fusion protein consisting of the morpholino-targeted sequence in frame with enhanced green fluorescent protein (EGFP). Embryos injected with the *LvEndo16-M*-GFP construct alone (a) or in combination with the standard control morpholino (b) produced a strong green fluorescent signal. In contrast, there was no green fluorescence associated with embryos coinjected with the LvEndo16 morpholino (c). Embryos injected with the LvEndo16 morpholino displayed an abnormal phenotype, as described in Figure 2. N > 25 for each treatment.

used cell type-specific monoclonal antibodies to determine if cellular differentiation was normal despite the morphological abnormalities elicited by the LvEndo16 morpholino. After ~24 h or 72 h in culture, we fixed embryos injected with the LvEndo16 morpholino or the standard control morpholino and stained them with the antibodies Endo1, EctoV, 1d5 or SMC-1. Endo1 is a protein of unknown function that is expressed in the region of the archenteron that corresponds to the prospective midgut and hindgut of the pluteus larva shortly after the onset of gastrulation (Wessel & McClay 1985). Endo1 expression was detected in the archenteron of embryos injected with the standard control morpholino after ~24 h in culture (Fig. 6c). The staining persisted in the midgut and hindgut of the pluteus larva (Fig. 6a). Initially, there was virtually no staining in Endo16-deficient embryos (Fig. 6d). Interestingly, Endo1 expression was eventually restored and was associated with the small archenteron-like structures that formed in some of the Endo16-deficient embryos (Fig. 6b). Even those Endo16-deficient embryos without small archenteron-like structures had discrete regions of staining by ~72 h.

The antibody 1d5 recognizes MSP-130, another glycoprotein that is expressed by PMC upon their ingression into the blastocoele during gastrulation (Anstrom *et al.* 1987). After ~24 h in culture, staining was associated with the ventrolateral clusters of PMC in control embryos (Fig. 6e). An equivalent level of staining was detected in the blastocoele of Endo16-

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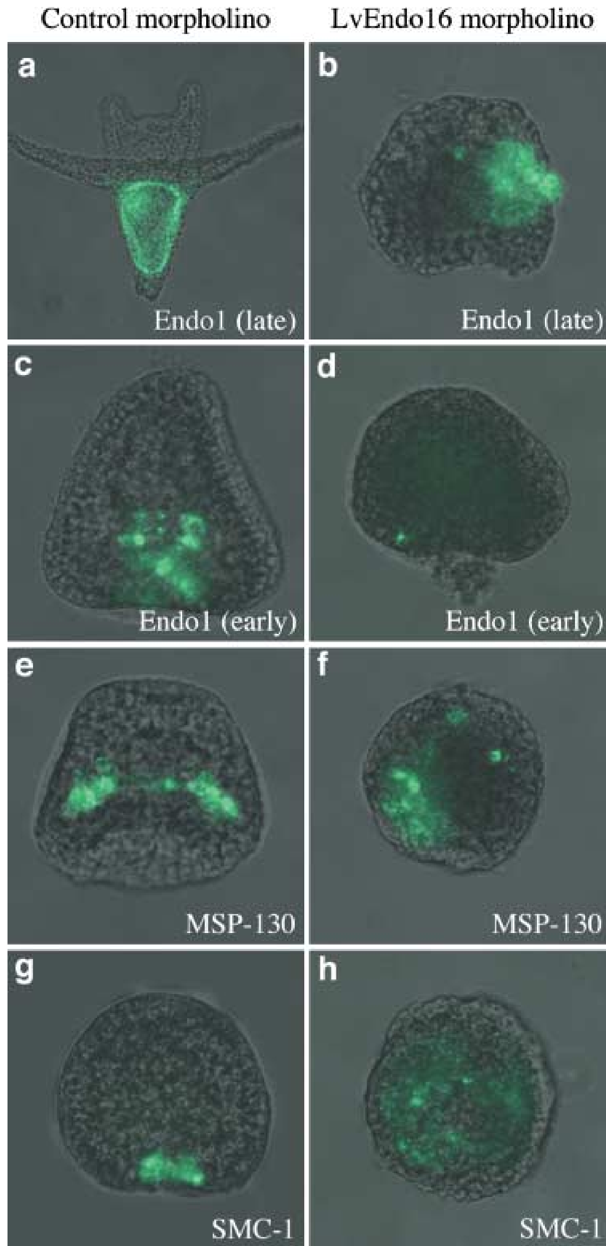

Fig. 6. Antibody staining of embryos injected with morpholinos. Embryos were stained with cell type-specific antibodies and observed under a fluorescent microscope. After ~24 h, embryos injected with the standard control morpholino displayed normal patterns of staining with antibodies that recognize Endo1, MSP-130 and SMC-1 (c, e, g). MSP-130 and SMC-1 expression also was detected in embryos injected with the LvEndo16 morpholino but the staining occurred in a random pattern (f, h). After ~24 h, embryos injected with the LvEndo16 morpholino had little or no staining with the antibody that recognizes Endo1 (d). By ~72 h, Endo1 expression was restored in some of the LvEndo16-deficient embryos at a level that was comparable to control embryos (a, b).

deficient embryos although the distribution of cells appeared to be random (Fig. 6f). That is, the staining was asymmetrical and did not appear as two discrete populations of cells as they do during normal development. 1g8, another antibody that recognizes MSP-130, produced similar results (data not shown).

We also stained the embryos with an antibody that recognizes SMC-1, which is a protein of unknown function that is expressed by presumptive SMC (Sweet *et al.* 1999). After ~24 h in culture, SMC-1 expression was detected at the vegetal plate of control embryos (Fig. 6g). The expression of SMC-1 in Endo16-deficient embryos was similar to that of MSP-130. Specifically, there was a random pattern of SMC-1 expression within the blastocoele of Endo16-deficient embryos, although the level of staining appeared to be normal as compared to embryos injected with the standard control morpholino (Fig. 6h).

Finally, we stained the embryos with an antibody that recognizes EctoV, a glycoprotein associated with the hyaline layer (Coffman & McClay 1990). EctoV expression becomes localized to the oral ectoderm during gastrulation (Coffman & McClay 1990). After ~24 h in culture, EctoV expression was detected throughout the oral ectoderm of control embryos (data not shown). It was difficult to assess the polarity of EctoV expression in Endo16-deficient embryos. Preliminary evidence suggests that levels of EctoV expression were equivalent to control embryos (data not shown). We suspect that the differentiation of aboral ectoderm was not affected by the LvEndo16 morpholino, although we did not confirm this because there is not a reliable antibody to detect Spec1 and other markers of aboral ectoderm in *L. variegatus*.

Discussion

Development of the digestive system is a multistep process that begins with the specification of endodermal precursors, well before gastrulation begins. In particular, a fate map revealed that there are approximately 155 cells in the center of the vegetal plate that become specified to form endoderm (Ruffins & Ettensohn 1996). Their commitment to this fate occurs shortly before gastrulation. Gastrulation begins with the primary invagination of the vegetal plate to form a short pocket of epithelium that contains both endoderm precursors and SMC. Several mechanisms appear to be involved in this primary invagination including changes in cell shape, cell adhesion and cytoskeletal organization. A secondary invagination that involves cell proliferation as well as cell rearrangement results in extension of this tissue to form a structure known as the archenteron. The

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archenteron continues to elongate with the aid of SMC that extend filopodia toward the overlying ectoderm. In particular, the filopodia receive cues from the overlying ectoderm and help pull the endoderm across the blastocoele. The ectoderm and endoderm tissues eventually fuse with one another at this 'target site' corresponding to the mouth of the embryo. The endoderm subsequently differentiates into a digestive system with a foregut, midgut and hindgut (reviewed by Wessel & Wikramanayake 1999).

A variety of genes including *Endo1*, *Endo16*, *ECM18*, *LvN1.2*, *Msx*, *HpHnf3*, *SpFkh1*, *SpHmx* and *SpKrox1* serve as useful markers of the endoderm in sea urchin embryos (Wessel & McClay 1985; Wessel *et al*. 1989; Berg *et al*. 1996; Harada *et al*. 1996; Wang *et al*. 1996; Dobias *et al*. 1997; Luke *et al*. 1997; Martinez & Davidson 1997). In particular, *Endo16* has been used to monitor endoderm specification. Previous research demonstrated that there is an inductive signaling event during endoderm specification that requires cell-cell contact. Dissociated embryos exhibit reduced levels of *Endo16* expression, and *Endo16* expression is restored if the cells are allowed to reassociate for 24 h (Godin *et al*. 1997). However, the precise function of Endo16 and other proteins expressed in the endoderm has remained unclear. It has been hypothesized that Endo16 plays a role in cell adhesion based on the fact that it associates with the basolateral surface of endodermal cells (Soltysik-Espanola *et al*. 1994). Moreover, the predicted amino acid sequence indicates that there are two regions of clustered repeats, which have been shown to bind to calcium (Soltysik-Espanola *et al*. 1994). Many cell adhesion molecules are dependent on calcium or other ions in order to achieve a stable conformation (Maurer *et al*. 1996).

Our data support the idea that Endo16 facilitates cell-cell and/or cell-ECM interactions during gastrulation. Specifically, Endo16-deficient embryos fail to undergo gastrulation and the blastocoele becomes filled with dissociated cells. Moreover, endoderm differentiation was delayed in Endo16-deficient embryos as assayed by staining with an antibody that recognizes Endo1, a different gut-specific protein. One possibility is that the dissociated cells correspond to presumptive endomesoderm and they have failed to maintain contact with each other due to lack of Endo16 protein. Another possibility is that these cells failed to invaginate during gastrulation due to altered interactions with the ECM. It was previously shown that gastrulation and the expression of endoderm markers such as Endo1 is dependent on cell-ECM interactions (Wessel & McClay 1987; Benson *et al*. 1991; Burdsal *et al*. 1991; Burke *et al*. 1991; Govindarajan *et al*. 1995; Godin *et al*. 1997). For example, embryos treated with chemicals that interfere with collagen processing or β-aminoproprionitrile (β-APN), which inhibits the formation of a collagen triple helix, fail to undergo gastrulation (Wessel & McClay 1987; Godin *et al*. 1997). Integrin is required for gastrulation and may mediate the interaction between endoderm and the ECM (Marsden & Burke 1998; Wessel & Wikramanayake 1999). Interestingly, Endo16 contains an RGD sequence, suggesting the possibility of interactions with integrin. Experiments using short synthetic peptides containing the RGD sequence would be necessary to confirm that integrin serves as a receptor for Endo16 (Ruoslahti 1996). It is also possible that Endo16 serves to localize growth factors within the ECM because the predicted amino acid sequence indicates that it may be capable of ligand binding (Wessel & Wikramanayake 1999). Platelet-derived growth factor (PDGF) is one example of a growth factor that is required for gastrulation in *L. variegatus* and whose signaling activity is dependent on the ECM (Govindarajan *et al*. 1995; Ramachandran *et al*. 1995).

The effect of the LvEndo16 morpholino appears to be transient, based on the fact that small archenteron-like structures expressing Endo1 eventually formed within the blastocoele. These data are consistent with previous studies highlighting the regulative nature of sea urchin development. For example, embryos can organize a new digestive system expressing Endo1 even after the entire archenteron is removed during gastrulation. Remaining descendants of the veg1 or veg2 tiers will expand their normal fates to compensate for the missing cells (McClay & Logan 1996). However, LvEndo16-deficient embryos did not survive beyond ~72 h of incubation despite this partial recovery. They displayed gross morphological abnormalities that prevented their development into pluteus larvae with a functional gut.

In summary, Endo16 probably plays a key role as a cell adhesion molecule during gastrulation in the sea urchin. Endo16 is a large extracellular protein that is expressed in the endoderm, consistent with this role. Although the transcriptional regulation of *Endo16* has been studied in immense detail, there has been little direct evidence regarding its precise function during development. Our data suggest that Endo16 may mediate cell-cell and/or cell-ECM interactions that are required for gastrulation. In the future, it would be informative to examine the adhesive properties of cells derived from Endo16-deficient embryos in an effort to validate this hypothesis. In particular, a centrifugation-based assay developed by McClay *et al*. (1981) could be performed to compare

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the adhesive properties of cells isolated from Endo16-deficient and control embryos. It would be interesting to investigate the function of specific domains to gain more insight into the role of this protein in cell adhesion and/or ligand binding.

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