Development 128, 4393-4404 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV5925

Sea urchin goosecoid function links fate specification along the animal-vegetal and oral-aboral embryonic axes

Lynne M. Angerer[1,*], David W. Oleksyn[1], Amy M. Levine[1], Xiaotao Li[2], William H. Klein[2] and Robert C. Angerer[1]

[1]Department of Biology, University of Rochester, Rochester, NY 14627, USA
[2]Department of Biochemistry and Molecular Biology, The University of Texas M.D. Anderson Cancer Center, and Graduate Program in Genes and Development, Houston, TX 77030, USA
*Author for correspondence (e-mail: langerer@mail.rochester.edu)

Accepted 13 August 2001

SUMMARY

We have identified a single homolog of *goosecoid*, *SpGsc*, that regulates cell fates along both the animal-vegetal and oral-aboral axes of sea urchin embryos. *SpGsc* mRNA is expressed briefly in presumptive mesenchyme cells of the ~200-cell blastula and, beginning at about the same time, accumulates in the presumptive oral ectoderm through pluteus stage. Loss-of-function assays with morpholine-substituted antisense oligonucleotides show that SpGsc is required for endoderm and pigment cell differentiation and for gastrulation. These experiments and gain-of-function tests by mRNA injection show that SpGsc is a repressor that antagonizes aboral ectoderm fate specification and promotes oral ectoderm differentiation. We show that SpGsc competes for binding to specific *cis* elements with SpOtx, a ubiquitous transcription activator that promotes aboral ectoderm differentiation. Moreover, SpGsc represses transcription in vivo from an artificial promoter driven by SpOtx. As SpOtx appears long before *SpGsc* transcription is activated, we propose that SpGsc diverts ectoderm towards oral fate by repressing SpOtx target genes. Based on the SpGsc-SpOtx example and other available data, we propose that ectoderm is first specified as aboral by broadly expressed activators, including SpOtx, and that the oral region is subsequently respecified by the action of negative regulators, including SpGsc. Accumulation of SpGsc in oral ectoderm depends on cell-cell interactions initiated by nuclear β-catenin function, which is known to be required for specification of vegetal tissues, because transcripts are undetectable in dissociated or in cadherin mRNA-injected embryos. This is the first identified molecular mechanism underlying the known dependence of oral-aboral ectoderm polarity on intercellular signaling.

Key words: Morpholino, Transcriptional repressor, Embryonic axes, Otx, β-catenin, Sea urchin

INTRODUCTION

Patterning of cell fates along the animal-vegetal (AV) and oral-aboral (OA) axes of the sea urchin embryo requires a signaling process that is initiated in the vegetal organizing center. Recent studies have established that the inductive processes that pattern mesoderm, endoderm and ectoderm fates along the AV axis are initiated by cell-autonomous activation of downstream components of the canonical Wnt pathway, i.e. β-catenin and TCF-Lef (Emily-Fenouil et al., 1998; Huang et al., 2000; Logan et al., 1999; Vonica et al., 2000; Wikramanayake et al., 1998). β-catenin/TCF-Lef activates, in at least one case directly (Howard et al., 2001), production of downstream transcription factors (SpKrl) and signaling ligands, including SpWnt8 (A. Wikramanayake and W. H. Klein, personal communication) and probably Delta (H. Sweet and C. Ettensohn, personal communication), which function in specifying vegetal fates.

The OA axis is established after fertilization by a mechanism that may involve polarized differences in redox potential (Coffman and Davidson, 2001). The point at which this axis is conditionally specified varies from the two-cell stage in *Strongylocentrotus purpuratus* (Cameron et al., 1989), the organism used in the studies reported here, to as late as the eight-cell stage in some other species (Kominami, 1988). OA polarity is displayed primarily in the ectoderm, by the differentiation of distinct oral and aboral epithelial cell types that are separated by a thin band of ciliated cuboidal cells. Although OA polarity is established after fertilization by a mechanism different from that which fixes the AV axis during oogenesis, patterning of different cell types arrayed along the OA axis nevertheless requires signaling from the vegetal pole. Animal halves of eight-cell embryos or intact embryos in which β-catenin function is blocked by injection of cadherin mRNA fail to differentiate aboral ectoderm or ciliary bands (Wikramanayake and Klein, 1997; Wikramanayake et al., 1998). Differentiation of these tissues can be rescued in animal half embryos by treating them with LiCl, which inhibits the β-catenin-destabilizing kinase, GSK3β, or by injection at the one-cell stage of mRNA encoding a stable

form of β-catenin (Wikramanayake et al., 1998). The fact that animal halves and cadherin-expressing embryos express the EctoV epitope, which also accumulates late in differentiated oral ectoderm (both facial epithelium and ciliary band), has led to the proposal that β-catenin-dependent signals are required specifically for aboral ectoderm differentiation (Wikramanayake et al., 1998).

In vertebrate embryos, activation of the canonical Wnt pathway leads to the establishment of a gastrulation organizing center that expresses the goosecoid transcription factor (Lemaire and Kodjabachian, 1996). The fact that ectopic expression of goosecoid can induce a second dorsal axis in *Xenopus* embryos suggests that it is a critical upstream regulator of dorsal fate in this embryo (Cho et al., 1991). Echinoderms are a sister clade to the chordates, whose embryos have been shown to use a number of molecular pathways employed in vertebrate embryos. In addition to the molecules noted above, BMP2/4, whose function is antagonized by goosecoid in *Xenopus* embryos, plays an important role in ectoderm specification in sea urchin embryos (Angerer et al., 2000). These considerations suggest that goosecoid might also play an important role in early sea urchin development. We present our characterization of an *S. purpuratus goosecoid* homolog, *SpGsc*, and our investigations of its role in sea urchin embryogenesis. We have determined its time and sites of expression, characterized phenotypes produced in loss-of-function and misexpression experiments and determined whether its expression depends on the canonical Wnt pathway. Our data show that SpGsc is required for gastrulation and subsequent differentiation of endoderm and pigment cells, one of the derivatives of secondary mesenchyme. SpGsc also plays a central role in patterning cell fates along the OA axis, in that its restricted expression in oral ectoderm is required to repress gene(s) that promote aboral ectoderm differentiation. The data presented here support a model in which SpGsc function links patterning of cell fates along the AV and OA axes of sea urchin embryos.

MATERIALS AND METHODS

Embryo and single-cell cultures

Adult sea urchins (*S. purpuratus*) were obtained from Charles M. Hollahan (Santa Barbara, CA). Embryos were cultured (Angerer and Angerer, 1981) and cell separation experiments were carried out (Reynolds et al., 1992) essentially as described previously. Blastomeres were resuspended in Ca^{2+}-free seawater and cultured in spinner flasks as described (Hurley et al., 1989).

Cloning and construct preparation

Degenerate primers representing sequences conserved in the homeodomains of *Xenopus* and *Drosophila* goosecoid proteins were used with a very early blastula random-primed cDNA library template to amplify the homologous cDNA. Full-length cDNA containing a 320 amino acid residue open reading frame (GenBank Accession Number, AF315231) was generated by 5′ and 3′ RACE (Life Technologies). *SpGsc* cDNA was inserted into Tclone, derived from the plasmid vector, pSp64T (Angerer et al., 2000), and synthetic mRNAs were transcribed with Sp6 RNA polymerase (Sp6 mESSAGE mACHINE, Ambion) from templates truncated with *Xba*I. The SpGsc-VP16 fusion construct was prepared as follows. The SpGsc DNA-binding domain (DBD) was obtained by PCR from the full-length cDNA clone described above using forward and reverse primers containing *Sac*I and *Not*I sites (underlined below), respectively:

forward, 5′CCC CGA GCT CTT GGT GAT *GGA ATT* CAA GAG AAA GAG GCG ACA;

reverse, 5′ CCC CGC GGC CGC TTA CCG TTT CTG CTT CCT C 3′.

This fragment was inserted into Tclone between the corresponding restriction sites and the VP16 transcriptional activation domain from clone pCS2-VP16ΔβXtcf-3 (corresponding to amino acid residues 411 to 490) (Vonica et al., 2000), which was contained on an *Eco*RI fragment, was inserted upstream of the SpGsc DBD at the *Eco*RI site in the forward primer (italics).

Hybridization assays

Blots of genomic DNA digested with either *Eco*RI or *Rsa*I were prepared as described previously (Yang et al., 1989a) and probed with random-primed cDNA sequence encoding the homeodomain and 22 additional 5′ amino acid residues in a solution containing 5×SSPE (0.75 M NaCl, 20 mM sodium phosphate, pH 7.4, 5 mM trisodium EDTA), 0.1% sodium pyrophosphate, 5×Denhardt's (0.5% polyvinylpyrrolidone, 0.5% bovine serum albumin and 0.5% Ficoll), 0.5% sodium dodecyl sulfate at 60°C and washed with 0.15×SSPE, 0.1% SDS at 45°C, which corresponds to a low stringency (T_m–45°C). RNA blotting was carried out as described previously (Howard et al., 2001) with 2 μg of mesenchyme blastula polyA$^+$RNA and the blot was washed at moderate stringency (T_m–35°C; 0.1×SSPE, 55°C). For RNase protection assays, total RNA (10 μg) from normal or dissociated embryos was purified with TRIzol reagent (Gibco BRL, Bethesda, MD) and hybridized to probes for *SpGsc* (1×10^8 cpm/μg), *Spec2a* (1×10^8 cpm/μg) (Hardin et al., 1988) or *SpHE* (1×10^7 cpm/μg) (Wei et al., 1999). Hybridization and analysis of RNase-resistant fragments were as described previously (Yang et al., 1989a). We used a radioactive method for in situ hybridization assays because it provides greater sensitivity and reproducibility for early embryonic stages than we have been able to achieve with whole-mount methods. Sections (5 μm) of embryos at selected developmental stages were hybridized with ^{33}P-labeled RNA probes for *SpGsc* (2.5×10^5 dpm/ng) as described previously (Angerer et al., 1987).

Generation of a glutathione-S-transferase (GST)-SpGsc fusion protein

A synthetic SpGsc protein was used to determine DNA-binding properties. To generate the pGST-GSC construct, pGEX-KG vector (Amersham Pharmacia Biotech, NJ) was digested with *Bam*HI and *Not*I. The *SpGsc* cDNA coding sequence was amplified by PCR using primers containing *Bam*HI and *Not*I sites:

Gsc-Bam forward, TGG GAT CCT GGA CTA TTA TCT CCC CGA CGT C;

Gsc-Not reverse, CGA TGC GGC CGC GGC GAG GAG ACC CCG ATG GTG AG.

The digested PCR fragment was ligated into pGEX-KG to produce the pGST-GSC construct. The fusion protein was expressed in *E. coli* BL21 cells. The GST-SpGsc fusion protein was purified using the GST Purification Module from Amersham. Briefly, an overnight culture derived from a single colony was diluted 100-fold with 500 ml NZY medium and incubated for 3 hours at 37°C. IPTG was added to 0.2 mM and the culture was incubated for an additional 2 hours at 37°C. Cells were harvested, resuspended in phosphate-buffered saline (PBS) with 1% N-laurylsarcosine (sarkosyl) and sonicated on ice for 1 minute (power level 4, 50% duty cycle). The lysate was clarified and Triton X-100 was added to the supernatant to a final concentration of 2%. Washed glutathione-agarose bead suspension (0.5 ml; 50% v/v in PBS) was added, and the lysate was incubated at room temperature on a shaker for 30 minutes. The beads were transferred to a supplied column and washed six times with ice-cold PBS. The fusion protein was eluted from the beads with 10 mM reduced glutathione and subsequently dialyzed into storage buffer (50 mM Hepes, pH 7.4, 100

mM KCL, 1 mM DTT, 1 mM PMSF, 10%(v/v) glycerol). The GST moiety on the SpGsc-GST fusion protein was removed by proteolytic digestion with Factor Xa for 10 hours at room temperature.

Electromobility shift assay (EMSA)

EMSA was performed as described previously (Yuh et al., 2001). The reactions contained 20-50 ng of the GST-SpGsc fusion protein or SpGsc protein with the GST moiety removed, 1×10^4 cpm of ^{32}P-end-labeled oligonucleotide probe, 100 ng of the indicated competitor, and 0.5 μg of poly(dI-dC) in a final volume of 15 μl 1×EMSA buffer (12% glycerol, 20 mM Hepes (pH 7.9), 5 mM $MgCl_2$, 100 mM KCl, 1 mM DTT) for 20 minutes at 4°C. DNA-protein complexes were resolved in a 5% polyacrylamide gel in 0.5×TBE and signals from the dried gels were recorded by a phosphorimager (Molecular Dynamics: Image Quant).

Injection of mRNAs and morpholino oligonucleotides.

Constructs for in vitro transcription of mRNAs were verified by sequencing. Synthetic mRNAs were suspended in 30% glycerol, quantitated by spectrophotometry and by gel electrophoresis, and microinjected as described previously (Angerer et al., 2000). Either 2×10^5 or 6×10^5 RNA molecules were injected into each egg. Morpholine-substituted oligonucleotides complementary to nucleotides –30 to –6 with respect to the translation start site of *SpGsc* mRNA and a control morpholino were obtained from Gene Tools (Corvallis, Oregon) and dissolved in diethylpyrocarbonate-treated water at a concentration of 8 mM. This stock solution was diluted to either 200 or 400 μM and 2 pl were injected to give a final concentration in the egg of 2-4 μM.

Promoter assay

A promoter containing multimerized *SpOtx cis* elements linked to the *CAT* (chloramphenicol acetyl transferase) reporter gene (5C) (Mao et al., 1996) and synthetic *SpGsc* mRNA were microinjected into one-cell sea urchin zygotes. CAT assays were carried out as described previously (Wei et al., 1995).

Immunostaining and microscopy

Embryos were fixed in artificial sea water containing 4% paraformaldehyde and stained with a polyclonal antibody against SpSoxB1, with a monoclonal antibody against a PMC-specific epitope (6e10, kindly supplied by Dr Chuck Ettensohn, Carnegie Mellon University) as described previously (Kenny et al., 1999), with Sp1 monoclonal antibody obtained from the Developmental Biology Hybridoma Bank (Dieter Soll, University of Iowa), and with polyclonal antibodies against Spec1 (Carpenter et al., 1984) and EctoV (Coffman and McClay, 1990) as described previously (Angerer et al., 2000). Fluorescent signals were captured by sequential scanning using a LeicaTS confocal microscope.

RT-PCR

RNA from 24-hour embryos injected with glycerol (control), synthetic mRNAs or morpholinos was purified with TRIzol reagent (Gibco BRL, Bethesda, MD), DNase I digestion, organic extraction and ethanol precipitation. One-step RT-PCR (Advanced Biotechnologies, Surrey, UK) using the primers listed below was employed to amplify *SpGsc*, *Endo16*, *BMP2/4*, or 12S mitochondrial rRNA sequences (Mito) as a load control for embryo RNA.

SpGsc forward primer: CCT GAG TAT CAC TTA GCT GCC
SpGsc reverse primer: TCG TTC CTC TTT GAG GTC GA
Endo16 forward primer: AAC AAG GTG CGT GCG GCT GC
Endo 16 reverse primer: GCT GGG GCG AGC ACA TTA TT
Mito forward primer: ACT CTC TCC TCG GAG CTA TA
Mito reverse primer: GTA TAA TTT TTG CGT ATT CGG C
SpBMP2/4 forward primer: 5 CAG GCC TAC TAT TGT CGC
SpBMP2/4 reverse primer: GGT ACT AGT GCT GGG TTG

Signals were compared within the linear phase of amplification, as verified by analyzing samples collected at different cycles. After electrophoresis of the samples through 6% polyacrylamide gels in TBE (0.1 M Tris, 0.1 M sodium borate, 2 mM EDTA, pH 8.3), signals were quantitated by phosphorimagery.

RESULTS

Isolation and characterization of the *SpGsc* cDNA sequence

Full-length *SpGsc* cDNA was isolated as described in Materials and Methods. It encodes a predicted peptide that contains two conserved regions corresponding to the homeodomain (Fig. 1A) and the goosecoid/engrailed homology (GEH) domain (Fig. 1B). Alignment of the homeodomain sequence with homologous sequences from other organisms indicates clearly that the closest relative to SpGsc is *Drosophila* Gsc (Accession Number, S70617), followed by homologs in the polychaete annelid (*Platynereis dumerilii*; Accession Number, CAC19336) and amphioxus (*Branchiostoma floridae*; Accession Number, AAF97935) (Neidert et al., 2000). Interestingly, most of the sequence divergence between these homeodomains and those of vertebrate Gsc proteins (Fig. 1A, boxed sequences) maps to the

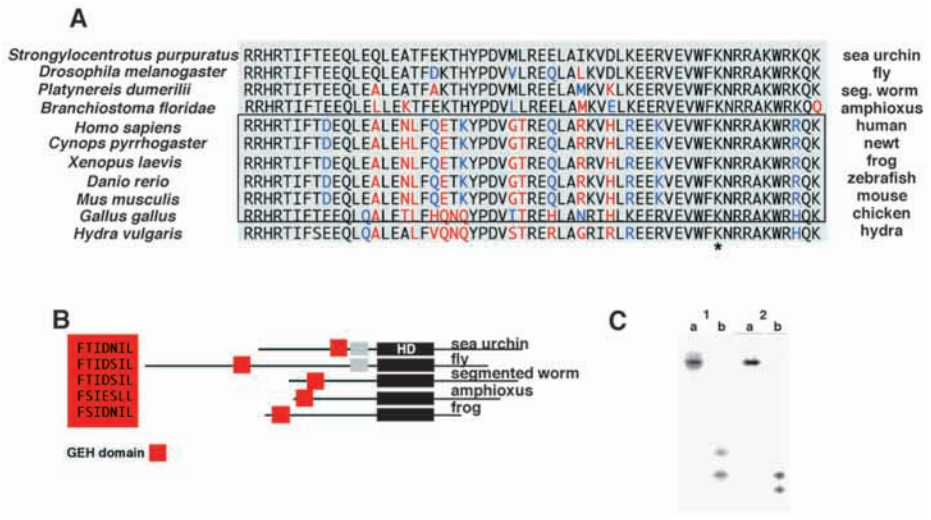


Fig. 1. (A) Goosecoid homeodomain sequence alignment. Red and blue residues indicate nonconservative and conservative amino acid substitutions, respectively. The asterisk indicates K50 a residue found in all bicoid-class homeodomain-containing proteins that is essential for binding to cognate *cis* elements. Vertebrate sequences are contained within the black rectangle. (B) Representative goosecoid proteins are aligned with respect to their homeodomain (HD) sequences. The relative positions of the conserved goosecoid-engrailed homology (GEH) domain that mediates repression are indicated by red boxes. An additional sequence that is similar between SpGsc and *Drosophila* Gsc is shown in gray. (C) Blot of sperm DNA from two individuals that has been digested with either *Eco*RI (a) or *Rsa*I (b) and hybridized at reduced stringency with a ^{32}P-labeled probe representing the homeodomain sequence (see Materials and Methods for details).

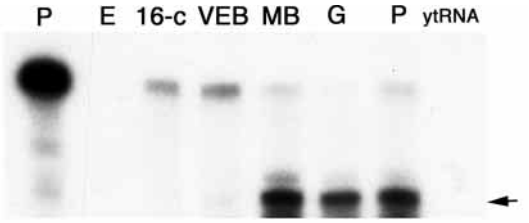


Fig. 2. RNase protection assays show that *SpGsc* transcripts accumulate at mesenchyme blastula (MB), gastrula (G) and pluteus (P) stages. A negative control is provided by hybridization to yeast tRNA (ytRNA). Unhybridized probe (P), egg (E), 16-cell stage (16-c), VEB (very early blastula, ~150 cells). The arrow indicates the band produced by the hybridized probe; trace amounts of undigested probe persist in some of the samples.

central region; both N- and C-terminal regions are extremely well conserved among all the members of this family. Because sea urchins are evolutionarily much more closely related to vertebrates than to *Drosophila*, and because multiple *Gsc* genes have been identified in both chickens and mice, we investigated the possibility that a second sea urchin *Gsc* gene exists that is more closely related to those of vertebrates. However, no evidence for a second gene could be detected in blots of DNA from individual sperm samples that were probed with the *SpGsc* homeodomain sequence at low stringency (Fig. 1C). In addition, reduced stringency hybridization screens of an arrayed 20 hour blastula cDNA library did not identify other *Gsc*-like sequences, although they did recover *SpGsc* again, as well as other cDNAs encoding homeodomain-containing proteins distinct from the bicoid-like class of which *SpGsc* is a member. The strong conservation of the Gsc homeodomains among an echinoderm, an arthropod, a polychaete annelid and a cephalochordate suggests that this is the ancestral gene. We conclude that after the divergence of cephalochordates and vertebrates, this gene was duplicated several times in the vertebrate line, after which the ancestral gene was lost. Nevertheless, despite some sequence differences between the central regions of the ancestral and vertebrate Gsc homeodomains, these proteins probably remain functionally similar. For example, *Drosophila* Gsc can elicit secondary axes when ectopically expressed in *Xenopus* embryos (Goriely et al., 1996).

The GEH peptide sequence in *Drosophila* Gsc is required to bind a co-repressor that mediates the repressor activity of Gsc (Mailhos et al., 1998). As shown in Fig. 1B, this sequence is well conserved in SpGsc, in which it probably functions similarly because, as shown below, SpGsc also has repressor activity in sea urchin embryos.

SpGsc expression pattern during embryogenesis

SpGsc transcripts are not detectable in the maternal RNA population or during cleavage; they accumulate between the very early blastula and mesenchyme blastula stages and persist at similar levels in the pluteus larva (Fig. 2). Blots of polyadenylated RNA detect a single 3.6 kb mRNA species (data not shown). In situ hybridization verifies that *SpGsc* transcripts are absent from eggs (Fig. 3A,B), cleaving embryos and very early blastulae (data not shown), but are detectable in hatched blastulae (Fig. 3C-H). At the latter stage the major region of expression encompasses about one half of the

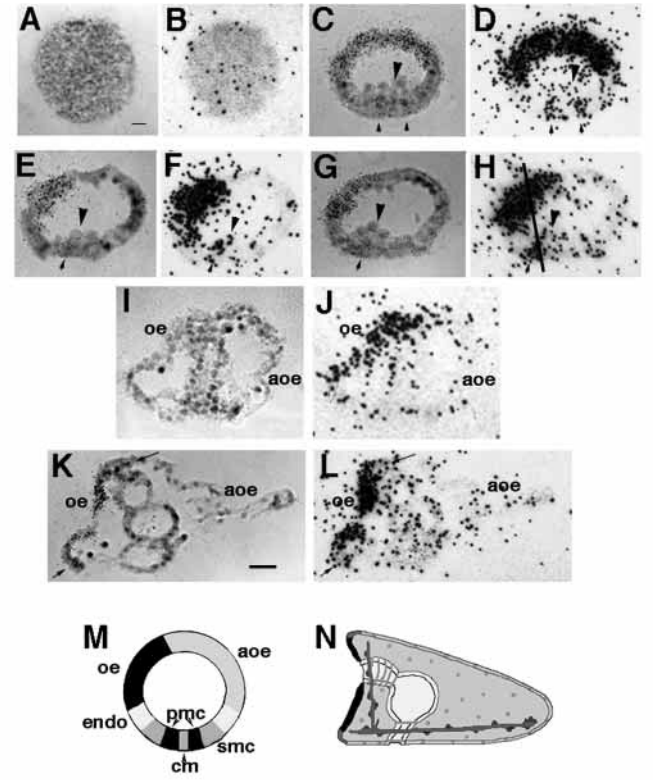


Fig. 3. In situ hybridization with ^{33}P-labeled antisense *SpGsc* probe shows that SpGsc mRNA accumulates in oral ectoderm throughout the mesenchyme blastula-pluteus period and transiently during blastula stages in presumptive mesenchyme cells in the vegetal plate. (A,C,E,G,I,K) Bright field images; (B,D,F,H,J,L) corresponding dark field images. (A,B) Egg; (C-H) early mesenchyme blastula; (I,J) gastrula; (K,L) pluteus. The arrowheads in C-H indicate recently ingressed primary mesenchyme cells. The black line in H indicates the approximate plane of section that would produce the hybridization pattern shown in C,D. The locations of oral and aboral ectoderm in I-L are marked with the labels oe and aoe, respectively. The distribution of SpGsc mRNAs is indicated in black in M (blastula) and N (pluteus). cm, coelomic mesenchyme; endo, endoderm; pmc, primary mesenchyme cells; smc, secondary mesenchyme cells. Embryos shown in C-L are oriented with the vegetal pole down. Scale bar: 20 μm.

presumptive ectoderm. The hybridization patterns of gastrulae (Fig. 3I,J) and plutei (Fig. 3K,L) identify this region as presumptive oral ectoderm. Lower signals are observed over a few cells in the vegetal plate region of early mesenchyme blastulae (Fig. 3D,F,H), which can be identified by the presence of a few adjacent, unlabeled, ingressed primary mesenchyme cells (PMCs). Comparison of adjacent sections (compare Fig. 3E,F with 3G,H) demonstrates the reproducibility of these vegetal signals, while hybridization patterns on fortuitously oriented sections (e.g. Fig. 3C,D) suggest that the positive cells are arranged in a torus around a few unlabeled cells at the vegetal pole. The size and position of the unlabeled region are characteristic of small micromere daughters, suggesting that the adjacent labeled cells are PMC precursors that have not yet ingressed into the blastocoel. *SpGsc* message accumulation in mesenchyme cells is transient and is downregulated as they enter the blastocoel. Based on their number and position, we favor the view that these cells

are PMC precursors, but we cannot exclude the possibility that some presumptive secondary mesenchyme cells also transiently express *SpGsc*. The early and late expression patterns are indicated by black shading in the diagrams in Fig. 3M,N.

SpGsc is required both for gastrulation and for establishing oral-aboral polarity

We examined the effects of loss of SpGsc function by morpholino-mediated inhibition of translation. We verified that the *SpGsc* morpholino inhibits translation in sea urchin embryos using the method described previously (Howard et al., 2001): *SpGsc* sequence complementary to the morpholino or the morpholino sequence itself (non-complementary) was inserted in the 5′UTR of the GFP cDNA template. Embryos simultaneously injected at the one-cell stage with the *SpGsc* morpholino and synthetic mRNA containing the complementary sequence did not produce detectable GFP, whereas embryos co-injected with mRNA containing the non-complementary sequence were brightly fluorescent (data not shown).

Loss of SpGsc function produced a striking phenotype. Most embryos failed to gastrulate or to differentiate endoderm, while a few produced only small gut rudiments (Fig. 4A, Table 1). As shown by RTPCR, 24-hour embryos from this population had greatly reduced levels of *Endo16* RNA (Fig. 4B), whose expression in the vegetal plate (presumptive endoderm and secondary mesenchyme) normally begins about the same time as does that of *SpGsc*. These embryos also lacked any vegetal derivatives labeled with monoclonal antibodies against EctoV, which labels foregut cells (Fig. 4A). The differentiation of pigment cells, a subset of secondary mesenchyme, was strongly suppressed: the embryo shown in Fig. 4C illustrates the maximum level of immunostaining with the Sp1 antibody, which recognizes an epitope expressed early in the differentiation of these cells, (Gibson and Burke, 1985); however, at this dose of SpGsc morpholino, most embryos lacked detectable Sp1 staining.

In addition to these defects in differentiation of vegetal cell types, embryos lacking SpGsc did not establish OA polarity, but instead assumed a radialized morphology that lacked definable oral and aboral territories separated by a ciliary band. Immunostaining reveals that all cells in the ectoderm express the aboral Spec1 marker. Little or no signal was detectable after immunostaining for the EctoV epitope, which is expressed at high levels and confined to the differentiated oral ectoderm of normal plutei (Fig. 4A). These observations demonstrate that SpGsc function is required for differentiation of endoderm, at least some, if not all, secondary mesenchyme, facial epithelium and ciliary band. By contrast, early differentiation of PMCs, as indicated by the presence of ingressed cells that stain specifically with the 6e10 antibody, is not affected (Fig. 4A, right; Fig. 4C).

SpGsc functions as a repressor in the oral ectoderm

The role of SpGsc within presumptive oral ectoderm could be to activate genes required for oral ectoderm fate, to repress those required for aboral fate, or both. The fact that the domain of expression of Spec1, a late aboral ectoderm marker, expands in the absence of SpGsc function and the presence of a GEH domain suggest a repressor function. One potential mechanism would be for SpGsc to antagonize the function of activators, such as SpOtx, another bicoid-class factor that is an essential early transcriptional activator in aboral ectoderm (Li et al., 1999; Mao et al., 1996). When SpOtx is converted to an active repressor by linking its DNA-binding domain to the engrailed repression domain and this protein is expressed in sea urchin embryos, accumulation of aboral ectoderm-specific markers is strongly suppressed (Li et al., 1999). Because Otx and Gsc have been shown to bind to the same *cis*-acting elements in other systems (Mailhos et al., 1998), it is likely that SpGsc can compete for binding at SpOtx target genes in oral ectoderm.

Table 1. Gut formation is suppressed in embryos injected with SpGsc-morpholino (Gsc-M)

	% embryos	
	Glycerol (*n*=49)	Gsc-M (*n*=79)
No gut	10	79
Small gut	23	17
Large gut	52	2
Dead embryos	15	9

If this hypothesis is correct, then misexpression of SpGsc should divert aboral ectoderm toward oral fate, while an activator consisting of the SpGsc DNA-binding domain linked to the VP16 activation domain should have the opposite effect. That this is, indeed, the case is shown in Fig. 5. The ectoderm of embryos injected with *SpGsc* mRNA expresses EctoV (oral), but little or no Spec1 (aboral). Conversely, embryos injected with *SpGsc-VP16* mRNA express predominantly Spec1. Thus, SpGsc and SpOtx-Eng behave similarly (Fig. 5, middle) (Li et al., 1999), as do SpGsc-VP16 and SpOtx (Fig. 5, bottom) (Mao et al., 1996).

The severity of the ectodermal defects caused by misexpression of SpGsc-VP16, as measured by the relative intensities of Spec1 and EctoV staining, appears to be greater than that produced by blocking SpGsc translation. This is not unexpected as SpGsc-VP16, when present at high levels throughout the embryo, is likely to have more transcriptional activation activity than that which is elicited by normal endogenous levels of SpOtx in the absence of SpGsc.

Both SpGsc loss-of-function experiments and the effects of SpGsc and SpGsc-VP16 misexpression support the idea that SpGsc can bind at SpOtx target sites and act as a competing repressor. To test this model directly, we performed EMSA to characterize SpGsc DNA binding properties in vitro, and transactivation assays to test the ability of SpGsc to directly compete with SpOtx function in vivo. EMSA showed that a bacterially produced GST-SpGsc fusion protein binds specifically to a DNA element contained on a 39 bp fragment (CII) previously shown to bind SpOtx and to mediate SpOtx activation of the aboral ectoderm-specific *Spec2a* gene (Mao et al., 1994). The CII probe also contains another *cis* element (OER) recently shown to mediate the binding of an oral ectoderm repressor unrelated to SpGsc (Yuh et al., 2001). As expected, the complex formed with GST-Gsc can be shifted with an anti-GST antibody (compare lanes 2 and 4 in Fig. 6A). This protein binds specifically to the Otx *cis* element, as competition with either the wild-type CII sequence or an oligonucleotide with mutations in the OER site effectively inhibited SpGsc-DNA complex formation (Fig. 6A, lanes 3, 7).

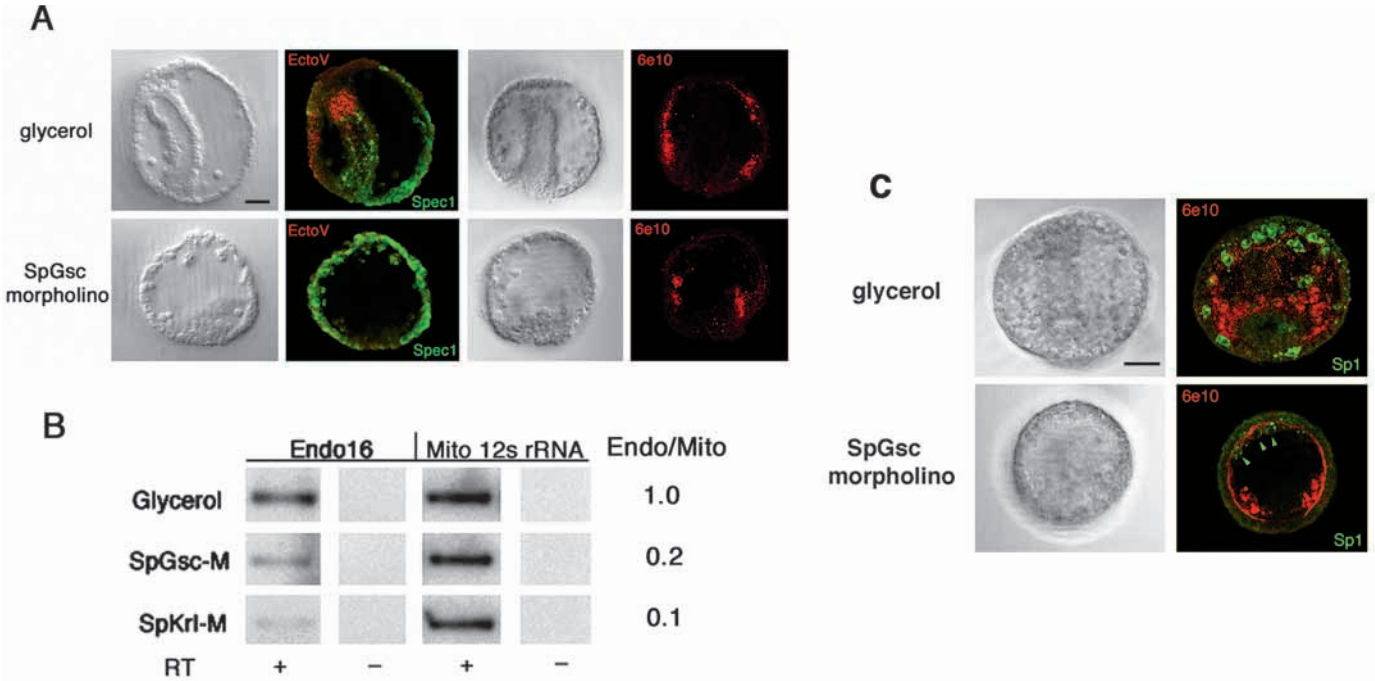


Fig. 4. Morpholino knockdown of SpGsc blocks oral endoderm and vegetal differentiation. (A) Confocal images of 3-day-old embryos injected either with 30% glycerol (top panels) or SpGsc morpholino in 30% glycerol (bottom panels). Embryos were stained either with antibodies against EctoV (red), which stains late oral ectoderm and foregut, and Spec1 (green), which stains aboral ectoderm at this stage (left side), or with antibody 6e10 that recognizes ingressed primary mesenchyme cells. (B) RT-PCR analysis of Endo16 mRNA levels in 24-hour embryos injected either with glycerol, or morpholinos against SpGsc (SpGsc-M) or SpKrl (SpKrl-M); RT, reverse transcriptase. Samples were analyzed at cycles 22 and 25 to verify that signals were compared during the linear phase of PCR amplification. Endo16 signals were normalized with respect to mitochondrial 12S rRNA values and these were set to a value of 1 for the positive control, which was RNA from glycerol-injected embryos. As SpKrl is required for Endo16 expression, SpKrl-M (morpholino) provides a negative control (Howard et al., 2001). (C) 2-day embryos that had been injected with glycerol (top) or the SpGsc morpholino (bottom) were stained with antibodies specific for the Sp1 epitope that is expressed on pigment cells (green) or for 6e10 (PMCs; red). Weakly Sp1-positive cells in SpGsc morpholino-injected embryos are indicated by arrowheads. Bars: 20 μm in A,C.

By contrast, sequences with mutated Otx sites incapable of binding to SpOtx were ineffective as competitors (Fig. 6A, lanes 5, 6). In particular, a mutation changing TAATCC to TAATTG, which is known to specifically affect binding of class K_{50} homeobox proteins, including Otx and Gsc (Hanes and Brent, 1991), did not compete for SpGsc binding at the Otx site (lane 5). To ensure that SpGsc was responsible for complex formation, we removed the GST moiety by proteolytic digestion with Factor Xa and repeated the EMSA. As expected, an SpGsc-DNA complex formed that was effectively competed by the CII wild-type oligonucleotide (Fig. 6A, lane 9) but not by the oligonucleotides with mutations in the Otx site (lanes 10, 11).

These experiments suggest that SpGsc binds to the Otx site with an affinity roughly comparable with that of SpOtx. This was confirmed by gel shift assays, which showed that the relative binding constant of SpGsc is within fourfold of that of SpOtx in vitro (3.1×10^3 versus 1.3×10^4). Direct competition between SpGsc and SpOtx for binding at the Otx site is demonstrated in Fig. 6B. In order to better distinguish between SpGsc- and SpOtx-DNA complexes by EMSA, we compared SpGsc with the GST moiety removed and the entire SpOtx-GST fusion protein. SpGsc alone formed a complex that was unaffected by the addition of an SpOtx antibody (Fig. 6B, lane 3). SpOtx-GST formed two lower mobility complexes that were both effectively supershifted with the SpOtx antibody (Fig. 6B, compare lanes 4 and 5). The faster migrating of the two SpOtx-GST complexes is likely to be a degradation product of the intact SpOtx-GST. When we fixed the amount of SpOtx-GST at 5.6 pmol and added increasing amounts of SpGsc from 2.8 to 19.9 pmol, we observed a decrease in the amount of SpOtx-GST complexes with a corresponding appearance of the SpGsc complex (Fig. 6B, lanes 6-8). About equal amounts of SpGsc and SpOtx-GST complexes were observed at an input ratio of 2 to 3 (lanes 7 and 8). In addition to the SpGsc complex, we observed a slightly slower migrating complex that behaved in a manner generally similar to the SpGsc complex and was dependent on added SpOtx-GST (Fig. 6B, lanes 6-8). Although the origin of this complex is unclear, it suggests an interaction between SpGsc and SpOtx-GST.

Conversely, when we kept SpGsc levels constant at 2.8 pmol and added increasing amounts of SpOtx-GST (1.4 to 11.2 pmol), we observed a decrease in the SpGsc complex and a corresponding increase in the SpOtx-GST complexes (Fig. 6B, lanes 9-13). Again, equimolar inputs of SpGsc and SpOtx-GST (lane 11) yield about three times more of the latter complex. Taken together, the EMSA results demonstrate that SpOtx and SpGsc are able to compete with one another at an Otx site.

To test the functional significance of the interaction between SpGsc and the Otx *cis* element in vivo, we used a transgene construct driven by a promoter whose activity is dependent on SpOtx *cis* elements (Mao et al., 1994). When increasing

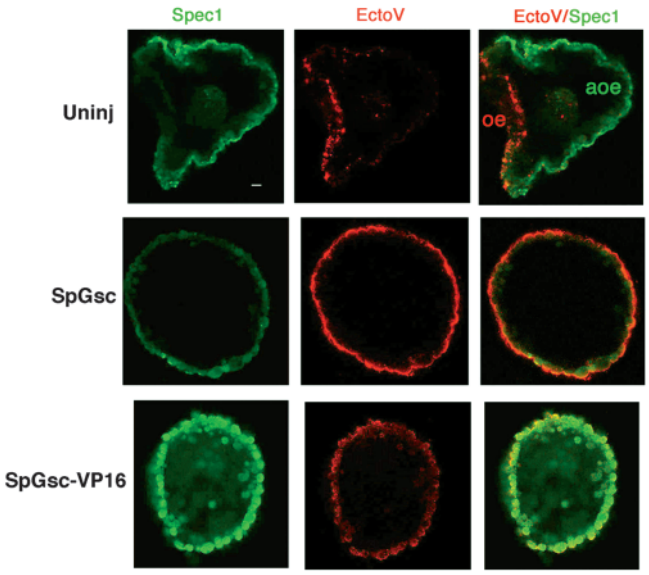


Fig. 5. Misexpression of SpOtx and SpGsc drive ectoderm toward aboral and oral fate, respectively. Confocal images of 3-day-old embryos double-stained with anti-EctoV and anti-Spec1 that (top panels) identify oral (red) and aboral (green) ectodermal territories in normal embryos at this stage. Sibling embryos were injected with mRNAs encoding either SpGsc (middle panels) or a fusion protein consisting of the SpGsc DNA-binding domain linked to the VP16 transcriptional activation domain (bottom panels). Separate Spec1 (left panels) and EctoV (center panels) signals are merged in the right panels. Misexpression of SpGsc promotes expression of the oral ectoderm marker in all ectodermal cells while misexpression of the VP16 fusion protein has the reciprocal effect, driving these cells to express predominantly the aboral ectoderm marker. All these confocal images were obtained at the same photomultiplier sensitivity. Scale bar: 10 μm.

amounts of *SpGsc* mRNA were co-injected with this construct at the one-cell stage, its promoter activity was progressively and strongly reduced (Fig. 6C). The straightforward interpretation of these results is that SpGsc downregulates the activity of this promoter by competition for binding at the SpOtx *cis* elements. An alternative mechanism, i.e. that SpGsc sequesters SpOtx in inactive heterodimers, is unlikely because in other systems formation of such heterodimers depends on the binding of both proteins to a palindromic paired homeodomain binding site (Mailhos et al., 1998), which is lacking in the target promoter used here.

Although SpOtx was identified as an activator of aboral ectoderm-specific genes, it accumulates in the nuclei of all ectoderm cells (Li et al., 1997). By contrast, SpGsc is expressed only in the oral facial epithelial region of the ectoderm where it can act to repress SpOtx target genes, as the data presented above demonstrate. This suggests that the ratio of SpGsc and SpOtx levels is an important factor in regulating oral versus aboral fates, and spatial regulation of *SpGsc* transcription is therefore an essential feature of oral ectoderm specification.

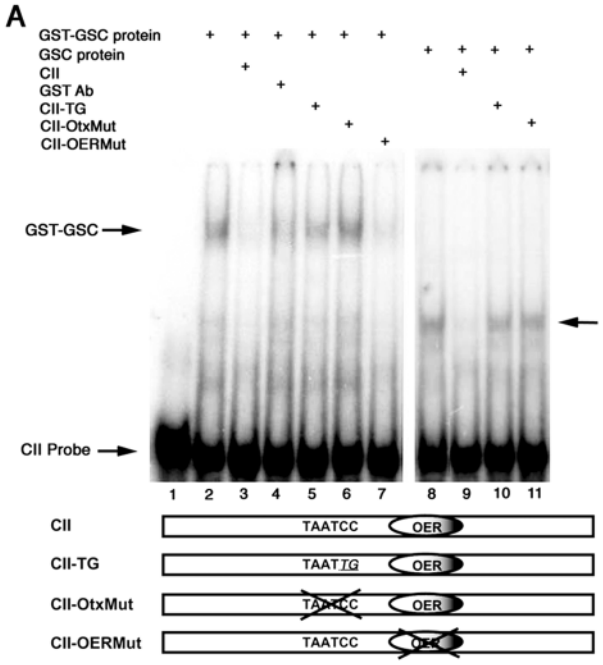
SpGsc transcription depends on cell-cell interactions that include β-catenin-dependent signals

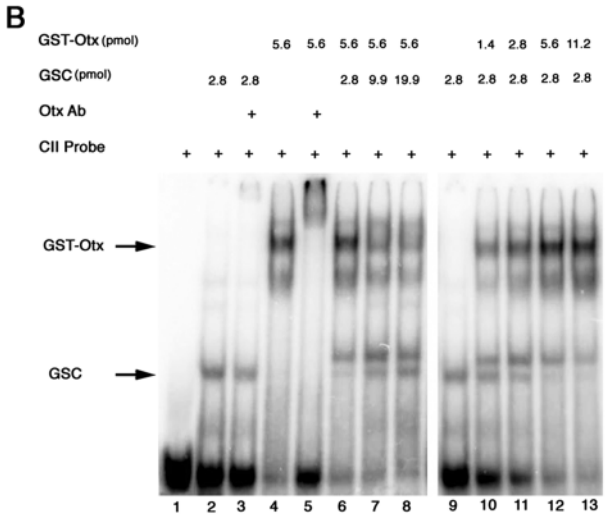
Differentiation of ectoderm requires signaling from vegetal cells. Thus, animal halves of embryos, or embryoids derived from egg animal hemispheres, remain as *dauerblastulae* and a variety of experimental manipulations that interfere with signaling by vegetal blastomeres also secondarily lead to radialization of the ectoderm (Angerer and Angerer, 2000). Such embryoids or embryos have been interpreted to differentiate as oral ectoderm because they express the late oral ectoderm marker, EctoV throughout. SpGsc is expressed in oral ectoderm beginning at hatching blastula stage, much earlier the EctoV synthesis can be detected, and it is required for establishing OA polarity. Therefore, it was of interest to test whether accumulation of *SpGsc* mRNA is activated cell autonomously in oral ectoderm and, if not, then whether these signals are dependent on β-catenin, which is a major component of the vegetal signaling mechanism.

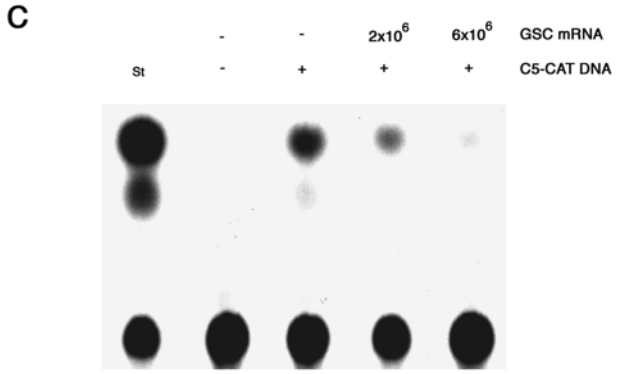
To test whether *SpGsc* expression is dependent on signals from other cells, transcript levels were measured by RNase protection in RNA from cells of embryos continuously dissociated beginning at the two-cell stage (Fig. 7A). No *SpGsc* mRNA was detectable in dissociated embryos by this sensitive assay, while control embryos showed the expected temporal pattern of accumulation. These results indicate that *SpGsc* expression in oral ectoderm, the major site of expression, is not activated cell autonomously, but they do not reveal whether the low level of transient *SpGsc* transcription in presumptive mesenchyme is sensitive to dissociation. In contrast to the sensitivity of *SpGsc* transcription to embryo dissociation, *SpHE* transcripts accumulate cell autonomously as previously reported (Ghiglione et al., 1993; Reynolds et al., 1992). Levels of 26S and 18S ribosomal RNAs in the stained gel demonstrate that the quality and recovery of RNA are equivalent in these samples.

To determine how late cell-cell interactions are required for *SpGsc* transcription, we dissociated embryos at progressively later cleavage and blastula stages and assayed *SpGsc* RNA levels when controls reached the gastrula stage (Fig. 7B). *SpGsc* message levels were strongly reduced even when embryos were dissociated as late as the ~200-cell early blastula stage, but not when they were left intact until the mesenchyme blastula stage (~500 cells), by which time *SpGsc* transcription has already begun in normal embryos. In this experiment, *SpHE* provides a contrasting control pattern of expression: *SpHE* transcripts are not present in 500-cell normal gastrulae (Reynolds et al., 1992), but they do persist to this stage in dissociated cells (Fig. 7B, dissociation at 2-, 32-, 64- and 200-cell stages), because interactions among cells are required for *SpHE* mRNA turnover (Ghiglione et al., 1993; Reynolds et al., 1992). These results indicate that the signaling required to initiate *SpGsc* transcription must continue until just a few hours before the gene is normally activated.

To determine whether *SpGsc* transcription in oral ectoderm requires nuclear β-catenin-dependent signals, *SpGsc* RNA levels were compared by RT-PCR in RNA of embryos injected with either glycerol or cadherin mRNA. As described previously (Howard et al., 2001; Logan et al., 1999; Wikramanayake et al., 1998), cadherin mRNA-injected embryos developed into epithelial spheres that lacked endoderm and mesenchyme (data not shown). The results of two experiments using different egg batches were in excellent agreement and are quantitated at the bottom of Fig. 7C, which also shows the primary data for experiment 2. In both cases,







controls in which reverse transcriptase was omitted were negative and signal intensities were consistent with sampling during the linear phase of amplification. These measurements

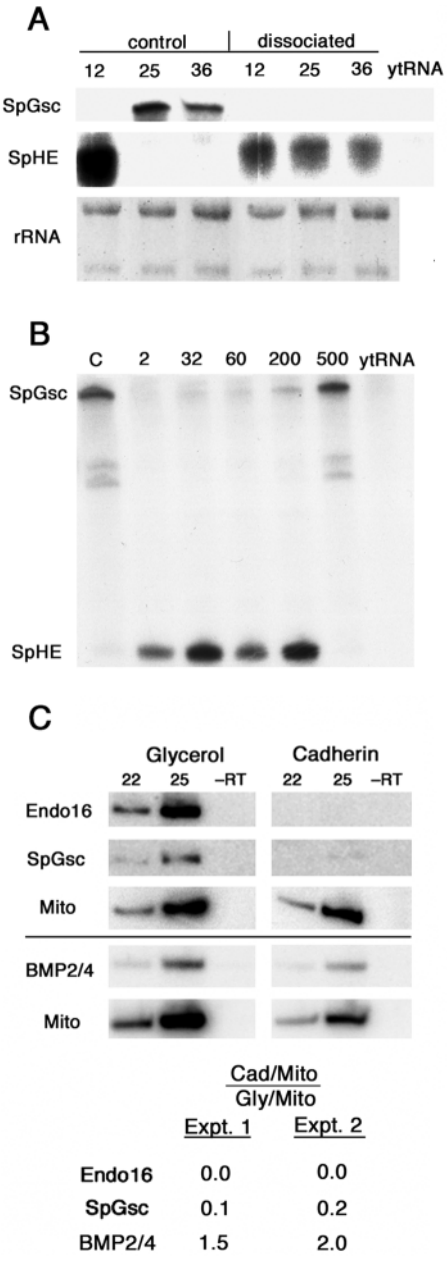
Fig. 6. SpGsc competes for binding at SpOtx *cis* elements. (A) SpGsc binds with specificity to an SpOtx site from the *Spec2a* promoter (contained on the CII fragment). Bacterially produced GST-GSC fusion protein purified by glutathione affinity chromatography formed a complex that was competable with the sequences containing an intact SpOtx *cis* element (lane 2 versus lanes 3 and 7), but not with those in which this element was altered by point mutation (lane 5) or by sequence replacement as defined by Yuh et al. (Yuh et al., 2001) (lane 6). Addition of GST antibody (lane 4) supershifted a significant fraction of this complex. GSC protein derived from GSC-GST also formed specific complexes (lane 8) as shown by competition reactions with the probe sequence (lane 9), and those containing mutated Otx elements (lanes 10,11). (B) SpGsc and SpOtx compete for binding to CII. GSC/CII complexes are shown in lanes 2 and 3. GST-Otx/CII complexes (lane 4) are supershifted with Otx antibody (lane 5). Reactions containing mixtures of GSC and GST-Otx were carried out under limiting probe concentrations. Lanes 6-8, constant amounts of GST-Otx were mixed with increasing quantities of GSC; Lanes 10-13, constant amounts of GSC were mixed with increasing amounts of GST-Otx. (C) SpGsc down regulates the activity of a promoter driven by SpOtx. Embryos (100) were injected with a promoter/CAT transgene construct and either no (–) or 2×10^6 or 6×10^6 molecules of SpGsc mRNA. St refers to a positive control reaction containing chloramphenicol acetyl transferase.

show that *SpGsc* RNA concentration is reduced between five- and tenfold in cadherin mRNA-injected 24-hour embryos. As expected, mRNA encoding the early vegetal plate marker, Endo16, is undetectable (Li et al., 1999; Wikramanayake et al., 1998).

To control for the formal possibility that lack of *SpGsc* expression results from a general arrest of ectoderm differentiation in cadherin-expressing embryos, we analyzed these embryos for accumulation of *BMP2/4* mRNA. In normal embryos, this message begins to be transcribed about the same time as *SpGsc* mRNA, and it accumulates throughout ectoderm with higher levels in the oral region (Angerer et al., 2000). As shown in Fig. 7C, *BMP2/4* mRNA accumulates to similar levels in cadherin-expressing and control embryos (compare BMP2/4 signals with mitochondrial rRNA load controls). We conclude that blocking β-catenin function does not generally inhibit activation of at least some relatively late ectoderm-specific genes.

DISCUSSION

The experiments presented here support the following major conclusions: (1) SpGsc acts downstream of nuclear β-catenin in sea urchin embryos, as demonstrated by its marked down regulation in embryos injected with cadherin mRNA; (2) as shown by morpholino antisense translational inhibition, SpGsc is required for gastrulation, for expression of the Endo16 vegetal marker, and for differentiation of pigment cells; and (3) SpGsc is required for differentiation of oral ectoderm and establishment of OA polarity, as also demonstrated by these loss-of-function experiments. Our results strongly suggest that SpGsc promotes oral fate by antagonizing aboral-specific gene functions. Thus, loss of SpGsc function in presumptive oral ectoderm causes it to express the Spec1 aboral ectoderm marker and misexpression of a transcription-activating



Cad/Mito / Gly/Mito	Expt. 1	Expt. 2
Endo16	0.0	0.0
SpGsc	0.1	0.2
BMP2/4	1.5	2.0

Fig. 7. Activation of SpGsc expression depends on cell-cell interactions and nuclear β-catenin. (A) *SpGsc* transcription requires cell-cell interactions. Cultures of single cells obtained from embryos dissociated in Ca^{2+}- and Mg^{2+}-free sea water at the two-cell stage were analyzed by RNase protection assays at very early blastula (12 hours), mesenchyme blastula (25 hours) and gastrula (36 hours) stages for the levels of *SpGsc* and *SpHE* (hatching enzyme) transcripts. RNA samples were checked for concentration and quality by electrophoresis through formaldehyde-containing gels (bottom panel). Negative controls were carried out with yeast tRNA (ytRNA). (B) Cell-cell interactions are required for *SpGsc* transcription until early blastula stages. RNase protection assays were carried out with a mixture of *SpGsc* and *SpHE* probes on samples isolated from cultures of embryos dissociated into single cells at either two-, 32-, 60-, 200- or 500-cell stages and assayed at the 600-cell gastrula stage. C refers to embryos that remained intact throughout the experiment. (C) *SpGsc* expression depends on the canonical Wnt signaling pathway because transcript levels are strongly reduced in 24-hour embryos injected with cadherin mRNA. RT-PCRs with primers specific for the *Endo16* vegetal plate marker or for *SpGsc* were sampled at either cycle 22 or cycle 25 and signals were quantitated by phosphorimagery and normalized for embryo number using the mitochondrial (Mito) 12S rRNA signal as described in the legend to Fig. 4. A similar analysis was carried out for the accumulation of *SpBMP2/4* message that is not inhibited by loss of β-catenin function. –RT refers to samples that were not reverse transcribed and Mito refers to detection of 12S mitochondrial rRNA to normalize for embryo loads among samples.

counterpart, SpGsc-VP16, diverts presumptive oral ectoderm toward aboral fate. As an example of this proposed mechanism, we showed that SpGsc binds with high affinity to *cis* elements recognized by a closely related factor, SpOtx, that activates *Spec2a* and is thought to function upstream of other genes required for aboral ectoderm differentiation (Li et al., 1999). Furthermore, a transgene promoter whose activity depends entirely on *SpOtx cis* elements is silenced in vivo by co-expression of SpGsc. Thus, SpGsc is essential for cell fate specification along both AV and OA axes.

SpGsc function in mesendoderm differentiation

Loss-of-function experiments demonstrate that *SpGsc* expression depends on β-catenin/TCF-Lef function and that it is essential for mesendoderm development. The observed phenotype indicates that SpGsc is required for early steps in differentiation of multiple mesendoderm derivatives. SpGsc knockdown embryos do not accumulate Endo16 mRNA, an early marker that initially is expressed throughout the vegetal plate of normal embryos. Pigment cells, the major SMC type do not differentiate as assayed by expression of the early Sp1 marker. No archenteron develops and no vegetal cells express the EctoV epitope that identifies differentiated foregut cells. Typically, SpGsc knockdown embryos have some ingressed cells that migrate away from the vegetal pole and display the 6e10 epitope, indicating that initial PMC differentiation is not blocked. Other cells clustered at the vegetal pole may be PMCs that have not yet begun to express the epitope or may represent other, arrested, vegetal cell types.

The vegetal domain in which we have detected *SpGsc* mRNA expression is restricted to a central torus in the vegetal plate that includes PMC precursors and perhaps some SMC precursors. As differentiation of other vegetal cells is affected, the outputs of SpGsc activity probably include non cell-autonomous signaling from either vegetal or oral ectoderm cells. The first, more traditional, possibility is that vegetal SpGsc is a downstream effector of the patterning mechanism initiated by vegetal β-catenin/TCF-Lef. An interesting alternative is that SpGsc function in oral ectoderm is required to activate signaling to the vegetal plate. Such a function could provide a feedback mechanism in which β-catenin-dependent signals cause expression of SpGsc in oral ectoderm, which then sends signals to mesendodermal tissues that promote their differentiation. However, because exogastrulae can develop relatively normal tripartite guts with pigment cells and muscle cells at their tip, such signals presumably would have to function transiently at the beginning of gastrulation. Of course, the potential mesenchymal and oral ectodermal roles of SpGsc in vegetal differentiation are not mutually exclusive and it should be possible to examine their separate contributions by constructing chimeras between macromere+mesomere tiers

and micromeres in which SpGsc translation is blocked in one component by a morpholino knockdown.

SpGsc function in oral ectoderm development

Activation of *SpGsc* in oral ectoderm clearly depends on nuclear β-catenin function. As differentiation of ectoderm is known to require vegetal signals, the most likely hypothesis is that expression in oral ectoderm is a consequence of the activity of the canonical, vegetal Wnt signaling pathway. While the possibility that β-catenin also functions in ectoderm nuclei cannot be excluded, it has never been detected there, even when these cells are induced to form endoderm. We found it surprising that *SpGsc* expression was blocked when nuclearization of β-catenin was inhibited by cadherin, because cadherin-treated embryos and embryos derived from animal halves express EctoV strongly throughout the ectoderm, which has consequently been interpreted to be oral ectoderm (Li et al., 1999). Thus, we anticipated that SpGsc would be up regulated in cadherin-treated embryos. The fact that it was downregulated leads us to suspect that EctoV accumulation is not always an indicator of oral ectoderm fate. Although EctoV accumulates specifically in differentiated oral ectoderm (both facial epithelium and ciliary band) at late stages, its initial pattern of zygotic synthesis, which begins at least several hours after SpGsc expression at late mesenchyme blastula stage (Coffman and McClay, 1990), is not known. While further studies are required to resolve this question, the relatively high and uniform concentration of EctoV in cadherin-expressing or animal-half embryos may alternatively reflect its abnormally prolonged, uniform synthesis in embryos in which aboral ectoderm specification is inhibited. We suggest it is not the case, however, that these embryos are arrested at a stage before *SpGsc* transcription begins, because they do transcribe *BMP2/4*, which is activated at the same time as *SpGsc* in normal embryos (Angerer et al., 2000).

If the nuclear β-catenin-dependent signals that activate *SpGsc* transcription in oral ectoderm come from vegetal blastomeres, then this mechanism would help explain why elaboration of oral-aboral patterning depends on vegetal signals. *SpGsc* message begins to accumulate in the presumptive oral territory of hatched blastulae only after major regions of the vegetal plate are thought to be conditionally specified but before the endoderm-ectoderm border is established (Angerer and Angerer, 2000). Our cell dissociation experiments show that the signaling required for SpGsc transcription must occur (or persist until) shortly before activation occurs. Therefore, activation of *SpGsc* in oral ectoderm most likely depends on signals originating from the adjacent conditionally specified endoderm. As oral ectoderm differentiation continues throughout gastrulation and the endoderm-ectoderm border remains subject to respecification during this time (McClay and Logan, 1996), continued signaling may be required relatively late in development. The persistence of high levels of SpGsc expression in oral ectoderm through the end of embryogenesis suggests that it acts within this territory to help maintain oral ectoderm fate.

Three steps in ectoderm patterning

Our observations on SpGsc function lead to a three-step model for ectoderm patterning. First, an autonomously acting set of animalizing transcription factors (ATFs) can drive initial specification of a pre-ectoderm state (Angerer and Angerer, 2000). Embryonic ectoderm passes only transiently through this state during cleavage in normal embryos. If embryos are artificially locked in this pattern of gene expression, as are animal-half embryos or embryos deprived of β-catenin/TCF-Lef function, they develop as classic *dauerblastulae*, which have a thickened wall at the animal pole and eventually express EctoV over most or all of their surface (depending on the species). Second, β-catenin-dependent signals emitted uniformly from the vegetal hemisphere then up regulate transcriptional regulators required for ectoderm to progress to conditional specification as aboral ectoderm. One of these regulators is SpOtx, which is present at similar concentration in all ectoderm nuclei (Li et al., 1997). This factor is present maternally and is likely to act upstream in the aboral ectoderm specification pathway. In addition to its accumulating in nuclei during cleavage stages, exogenously supplied SpOtx can rescue uniform expression of aboral ectoderm-specific genes in embryos injected with cadherin mRNA, including *actin CyIIIa*, which is thought not to be activated directly by SpOtx. In the third step, patterning of ectoderm to form separate oral and aboral territories requires activation of repressors of aboral ectoderm genes in the oral territory. This proposed sequence of events is consistent with previous observations that many genes ultimately expressed in only the aboral ectoderm are initially activated at the end of cleavage throughout most or all of the ectoderm (Kingsley et al., 1993; Yang et al., 1989a; Yang et al., 1989b). Only subsequently are these genes downregulated in oral ectoderm, beginning around mesenchyme blastula stage, which is the time when SpGsc begins to accumulate. Interestingly, the other spatial regulators of aboral ectoderm-specific genes identified to date (p3A2) (Kirchhamer and Davidson, 1996) (OER) (Yuh et al., 2001) also function as repressors in oral ectoderm rather than as spatially restricted activators in the aboral territory.

We have provided one mechanism for SpGsc function in oral ectoderm differentiation by demonstrating that it can compete with SpOtx to repress genes promoting aboral ectoderm fate. Thus, establishment of discrete ectoderm territories does not occur until competitive levels of repressor activities, such as that of SpGsc, are reached just after hatching blastula stage. When SpGsc is misexpressed precociously and ectopically by mRNA injection, it presumably displaces SpOtx from its target genes throughout the presumptive ectoderm, leading to suppression of aboral ectoderm differentiation. Conversely, overexpression of SpOtx effectively competes SpGsc function in oral ectoderm, thereby allowing continued transcription of aboral-specific genes (Mao et al., 1996). This remarkable ability of individual factors like SpGsc and SpOtx to drive differentiation towards a specific cell type may suggest that these factors function far upstream in the pathways that specify ectoderm tissues. We think a more likely alternative is that each of these factors is a member of a network of crossregulating activators and repressors, and that these networks can be manipulated by altering the levels of individual members. Thus, it will be of interest to identify other regulatory factors operating in these tissues.

A critical component of this model that remains to be identified is the activity that restricts *SpGsc* expression to the oral region. The specific activation of *SpGsc* in presumptive oral ectoderm demonstrates that a unique transcriptional territory

has been established there by about the 250-cell stage. This activity cannot depend on vegetal nuclear β-catenin, which exhibits no OA polarity. This polarizing component most probably arises from the poorly understood mechanism that initially specifies the OA axis. It might originate in the early redox gradient that predicts the alignment of this axis and has been shown to affect the activity of at least one transcriptional regulator (Coffman and Davidson, 2001). Although vegetal signals clearly are required for establishing OA polarity, it is not yet known whether these signals are instructive (i.e. the polarity is imposed from the vegetal cells) or permissive (i.e. uniform vegetal signals are required to implement an inherent OA polarity in the ectoderm). Evidence that a relatively early, vegetal OA polarity exists is that the Notch receptor is enriched on the apical surfaces of presumptive endoderm cells on the aboral side around the blastula stage (Sherwood and McClay, 1997). If the model we have presented is correct, then identification of the spatial regulatory elements of the *SpGsc* promoter should define the additional activities that specify oral ectoderm, bringing us much closer to understanding how the oral-aboral axis is established.

This work was supported by grants from NIGMS (EM25553) (R. C. A.), NICHD (HD22619 to W. H. K.) and the Robert A. Welch Foundation (G-0010 to W. H. K.). We would like to thank Athula Wikramanayake and Ruthie Zearfoss for generating the original *SpGsc* PCR product, and Drs Alan Vonica and Judy Venuti for a plasmid containing the VP16 domain.

REFERENCES

A**ngerer**, **L. M. and Angerer, R. C**. (1981). Detection of poly A+ RNA in sea urchin eggs and embryos by quantitative in situ hybridization. *Nucleic Acids Res.* **9**, 2819-2840.

Angerer, L. M. and Angerer, R. C. (2000). Animal-vegetal axis patterning mechanisms in the early sea urchin embryo. *Dev. Biol.* **218**, 1-12.

Angerer, L. M., Cox, K. H. and Angerer, R. C. (1987). Demonstration of tissue-specific gene expression by in situ hybridization. *Methods Enzymol.* **152**, 649-661.

Angerer, L. M., Oleksyn, D. W., Logan, C. Y., McClay, D. R., Dale, L. and Angerer, R. C. (2000). A BMP pathway regulates cell fate allocation along the sea urchin animal-vegetal embryonic axis. *Development* **127**, 1105-1114.

Cameron, R. A., Fraser, S. E., Britten, R. J. and Davidson, E. H. (1989). The oral-aboral axis of a sea urchin embryo is specified by first cleavage. *Development* **106**, 641-647.

Carpenter, C. D., Bruskin, A. M., Hardin, P. E., Keast, M. J., Anstrom, J., Tyner, A. L., Brandhorst, B. P. and Klein, W. H. (1984). Novel proteins belonging to the troponin C superfamily are encoded by a set of mRNAs in sea urchin embryos. *Cell* **36**, 663-671.

Cho, K. W., Blumberg, B., Steinbeisser, H. and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: the role of the *Xenopus* homeobox gene goosecoid. *Cell* **67**, 1111-1120.

Coffman, J. A. and McClay, D. R. (1990). A hyaline layer protein that becomes localized to the oral ectoderm and foregut of sea urchin embryos. *Dev. Biol.* **140**, 93-104.

Coffman, J. A. and Davidson, E. H. (2001). Oral-Aboral axis specification in the sea urchin embryo. *Dev. Biol.* **230**, 18-28.

Emily-Fenouil, F., Ghiglione, C., Lhomond, G., Lepage, T. and Gache, C. (1998). GSK3beta/shaggy mediates patterning along the animal-vegetal axis of the sea urchin embryo. *Development* **125**, 2489-2498.

Ghiglione, C., Lhomond, G., Lepage, T. and Gache, C. (1993). Cell-autonomous expression and position-dependent repression by Li+ of two zygotic genes during sea urchin early development. *EMBO J.* **12**, 87-96.

Gibson, A. W. and Burke, R. D. (1985). The origin of pigment cells in embryos of the sea urchin *Strongylocentrotus purpuratus*. *Dev. Biol.* **107**, 414-419.

Goriely, A., Stella, M., Coffinier, C., Kessler, D., Mailhos, C., Dessain, S. and Desplan, C. (1996). A functional homologue of goosecoid in *Drosophila*. *Development* **122**, 1641-1650.

Hanes, S. D. and Brent, R. (1991). A genetic model for interaction of the homeodomain recognition helix with DNA. *Science* **251**, 426-430.

Hardin, P. E., Angerer, L. M., Hardin, S. H., Angerer, R. C. and Klein, W. H. (1988). Spec2 genes of *Strongylocentrotus purpuratus*. Structure and differential expression in embryonic aboral ectoderm cells. *J. Mol. Biol.* **202**, 417-431.

Howard, E. W., Newman, L. A., Oleksyn, D. W., Angerer, R. C. and Angerer, L. M. (2001). SpKrl: a direct target of β-catenin regulation required for endoderm differentiation in sea urchin embryos. *Development* **128**, 365-375.

Huang, L., Li, X., El-Hodiri, H. M., Dayal, S., Wikramanayake, A. H. and Klein, W. H. (2000). Involvement of Tcf/Lef in establishing cell types along the animal- vegetal axis of sea urchins. *Dev. Genes Evol.* **210**, 73-81.

Hurley, D. L., Angerer, L. M. and Angerer, R. C. (1989). Altered expression of spatially regulated embryonic genes in the progeny of separated sea urchin blastomeres. *Development* **106**, 567-579.

Kenny, A. P., Kozlowski, D., Oleksyn, D. W., Angerer, L. M. and Angerer, R. C. (1999). SpSoxB1, a maternally encoded transcription factor asymmetrically distributed among early sea urchin blastomeres. *Development* **126**, 5473-5483.

Kingsley, P. D., Angerer, L. M. and Angerer, R. C. (1993). Major temporal and spatial patterns of gene expression during differentiation of the sea urchin embryo. *Dev. Biol.* **155**, 216-234.

Kirchhamer, C. V. and Davidson, E. H. (1996). Spatial and temporal information processing in the sea urchin embryo: modular and intramodular organization of the *CyIIIa* gene cis-regulatory system. *Development* **122**, 333-348.

Kominami, T. (1988). Determination of dorso-ventral axis in early embryos of the sea urchin, *Hemicentrotus pulcherrimus*. *Dev. Biol.* **127**, 187-196.

Lemaire, P. and Kodjabachian, L. (1996). The vertebrate organizer: structure and molecules. *Trends Genet.* **12**, 525-531.

Li, X., Chuang, C. K., Mao, C. A., Angerer, L. M. and Klein, W. H. (1997). Two Otx proteins generated from multiple transcripts of a single gene in *Strongylocentrotus purpuratus*. *Dev. Biol.* **187**, 253-266.

Li, X., Wikramanayake, A. H. and Klein, W. H. (1999). Requirement of SpOtx in cell fate decisions in the sea urchin embryo and possible role as a mediator of beta-catenin signaling. *Dev. Biol.* **212**, 425-439.

Logan, C. Y., Miller, J. R., Ferkowicz, M. J. and McClay, D. R. (1999). Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development* **126**, 345-357.

Mailhos, C., Andre, S., Mollereau, B., Goriely, A., Hemmati-Brivanlou, A. and Desplan, C. (1998). *Drosophila* Goosecoid requires a conserved heptapeptide for repression of paired-class homeoprotein activators. *Development* **125**, 937-947.

Mao, C. A., Gan, L. and Klein, W. H. (1994). Multiple Otx binding sites required for expression of the *Strongylocentrotus purpuratus Spec2a* gene. *Dev. Biol.* **165**, 229-242.

Mao, C. A., Wikramanayake, A. H., Gan, L., Chuang, C. K., Summers, R. G. and Klein, W. H. (1996). Altering cell fates in sea urchin embryos by overexpressing SpOtx, an orthodenticle-related protein. *Development* **122**, 1489-1498.

McClay, D. R. and Logan, C. Y. (1996). Regulative capacity of the archenteron during gastrulation in the sea urchin. *Development* **122**, 607-616.

Neidert, A., Panopoulou, G. and Langeland, J. (2000). Amphioxus goosecoid and the evolution of the head organizer and prechordal plate. *Evol. Dev.* **2**, 303-310.

Reynolds, S. D., Angerer, L. M., Palis, J., Nasir, A. and Angerer, R. C. (1992). Early mRNAs, spatially restricted along the animal-vegetal axis of sea urchin embryos, include one encoding a protein related to tolloid and BMP-1. *Development* **114**, 769-786.

Sherwood, D. R. and McClay, D. R. (1997). Identification and localization of a sea urchin Notch homologue: insights into vegetal plate regionalization and Notch receptor regulation. *Development* **124**, 3363-3374.

Vonica, A., Weng, W., Gumbiner, B. M. and Venuti, J. M. (2000). TCF is the nuclear effector of the beta-catenin signal that patterns the sea urchin animal-vegetal axis. *Dev. Biol.* **217**, 230-243.

Wei, Z., Angerer, L. M., Gagnon, M. L. and Angerer, R. C. (1995). Characterization of the *SpHE* promoter that is spatially regulated along the animal-vegetal axis of the sea urchin embryo. *Dev. Biol.* **171**, 195-211.

Wei, Z., Angerer, L. M. and Angerer, R. C. (1999). Spatially regulated SpEts4 transcription factor activity along the sea urchin embryo animal-vegetal axis. *Development* **126**, 1729-1737.

Wikramanayake, A. H. and Klein, W. H. (1997). Multiple signaling events specify ectoderm and pattern the oral-aboral axis in the sea urchin embryo. *Development* **124**, 13-20.

Wikramanayake, A. H., Huang, L. and Klein, W. H. (1998). beta-catenin is essential for patterning the maternally specified animal-vegetal axis in the sea urchin embryo. *Proc. Natl. Acad. Sci. USA* **95**, 9343-9348.

Yang, Q., Angerer, L. M. and Angerer, R. C. (1989a). Structure and tissue-specific developmental expression of a sea urchin arylsulfatase gene. *Dev. Biol.* **135**, 53-65.

Yang, Q., Angerer, L. M. and Angerer, R. C. (1989b). Unusual pattern of accumulation of mRNA encoding EGF-related protein in sea urchin embryos. *Science* **246**, 806-808.

Yuh, C. H., Li, X., Davidson, E. H. and Klein, W. H. (2001). Correct expression of *Spec2a* in the sea urchin embryo requires both Otx and other cis-regulatory elements. *Dev. Biol.* **232**, 424-438.