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Major regulatory factors in the evolution of development: the roles of *goosecoid* and *Msx* in the evolution of the direct-developing sea urchin *Heliocidaris erythrogramma*

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SUMMARY The transcription factors *Gsc* and *Msx* are expressed in the oral ectoderm of the indirect-developing sea urchin *Heliocidaris tuberculata*. Their patterns of expression are highly modified in the direct developer *Heliocidaris erythrogramma*, which lacks an oral ectoderm. We here test the hypothesis that they are large effect genes responsible for the loss of the oral ectoderm module in the direct-developing larva of *H. erythrogramma* as well as for the restoration of an overt oral ectoderm in *H.e.* × *H.t.* hybrids. We undertook misexpression/overexpression and knockdown assays in the two species and in hybrids by mRNA injection. The results indicate that dramatic changes of function of these transcription factors has occurred. One of these genes, *Gsc*, has the ability when misexpressed to partially restore oral ectoderm in *H. erythrogramma*. On the other hand, *Msx* has lost any oral function and instead has a role in mesoderm proliferation and patterning. In addition, we found that the *H. tuberculata Gsc* is up regulated in *H.e.* × *H.t.* hybrids, showing a preferential use of the indirect developing parental gene in the development of the hybrid. We suggest that *Gsc* qualifies as a gene of large evolutionary effect and is partially responsible for the evolution of direct development of *H. erythrogramma*. We discuss these results in light of modularity and genetic networks in development, as well as in their implications for the rapid evolution of large morphological changes in development.

INTRODUCTION

The oral ectoderm is a gene expression territory present in the pluteus larva of indirect-developing sea urchins. Interactions between the oral ectoderm and other territories is necessary for the proper formation of several other features of the feeding larva such as ciliary band, aboral ectoderm, and skeletogenic mesenchyme patterning (Armstrong et al. 1993; Wikramanayake et al. 1995; Ramachandran et al. 1997; Wikramanayake and Klein 1997; Tan et al. 1998; Zito et al. 1998; Di Bernardo et al. 1999; Angerer and Angerer 2003; Duboc et al. 2004). *Heliocidaris tuberculata* produces a well-defined oral territory that has the same expression of molecular markers as well as all morphological features of the oral territory defined in other sea urchins. This territory is not present as a discrete feature in the larva of the direct-developing species *Heliocidaris erythrogramma*, whose ectoderm is composed only of vestibular and extra-vestibular ectoderms (Raff and Sly 2000). It is significant that *H. erythrogramma* produces a vestibule, the site of juvenile urchin development, which is normally derived from the oral territory of indirect developing sea urchins. *H.e.* × *H.t.* hybrid embryos exhibit restoration of features of indirect development (Raff et al. 1999). This result indicated the presence of some dominant-acting factor or factors from the *H. tuberculata* genome acting to control the cellular differentiation and morphology of the hybrid embryos. One important aspect of this restoration was the presence of a well-defined oral ectoderm, presenting us with a target tissue for many aspects of the rescue of an indirect developing phenotype. The restoration of oral ectoderm is important in two respects. First, it indicates that genes with large phenotypic effects are involved in the evolutionary loss of pluteus features. Second, oral ectoderm is known to express regulatory genes required for oral ectoderm development and it functions in patterning of other tissues.

The existence of several transcription factors known to localize to the oral ectoderm in other indirect-developing sea urchins prompted a candidate gene search, which resulted in the cloning and characterization of three homeodomain containing transcription factors from the genus *Heliocidaris* (Zhou et al. 2003; Wilson et al. 2005). Two of these genes, *Gsc* and *Msx*, exhibit expression patterns indicating a possible role in the evolution of direct development (Wilson et al. 2005).

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Msx is a transcription factor implicated in a variety of developmental processes from tooth formation (Satokata and Maas 1994) to limb regeneration and development in vertebrates (Koshiba et al. 1998), as well as CNS and muscle formation in *Drosophila* (Isshiki et al. 1997; Nose et al. 1998). *SpMsx* is implicated by misexpression/overexpression studies to play a role in gastrulation and in skeletal, mesenchymal and ectodermal patterning in the indirect-developing sea urchin *Strongylocentrotus purpuratus* (Dobias et al. 1997). *Msx* expression in *H. tuberculata* is observed in oral ectoderm, as seen in *S. purpuratus* (Wilson et al. 2005). In contrast, the expression of *Msx* is mainly coelomic and vestibular ectoderm in *H. erythrogramma* larvae (Wilson et al. 2005). This switch in pattern of territory expression may represent an important evolutionary change in the direct developer.

SpGsc (*goosecoid*) encodes a powerful transcriptional repressor expressed in the oral ectoderm in *S. purpuratus*. Misexpression/overexpression in *S. purpuratus* represses aboral fate, allowing the entire ectoderm to take on an oral identity (Angerer et al. 2001). Additionally, both gastrulation and skeletal patterning appear affected in these animals (Angerer et al. 2001). The molecular knockout of *Gsc* by injection of morpholino oligonucleotides into *S. purpuratus* promote an aboral-like fate in all ectoderm (Angerer et al. 2001). The expression pattern of *Gsc* in *H. tuberculata* is like that of *S. purpuratus*, with an early and persistent expression within the oral territory (Wilson et al. 2005). The pattern of expression in *H. erythrogramma* is particularly intriguing, as *Gsc* appears to be expressed first in the entire ventral half of the larval ectoderm (Wilson et al. 2005). This ventral side has been proposed to represent the position of the now rudimentary oral field in *H. erythrogramma*, based on a number of morphological features (Emlet 1995; Byrne et al. 2001), as well as the localization of *Gsc* itself (Wilson et al. 2005). *Gsc* expression progressively resolves to the vestibular ectoderm on the left side, with oral expression diminishing (Wilson et al. 2005).

In this study, we experimentally test the functions of *Msx* and *Gsc* in the evolution of developmental mode. The genus *Heliocidaris* offers us a unique opportunity to resolve questions about the evolution of larval form. Both species are amenable to misexpression/overexpression assays as are *H.e.* × *H.t.* hybrids. Knockdown studies have also become feasible. The advent of morpholino oligos and their effectiveness in indirect developing sea urchins (Angerer et al. 2001) allow us to examine knockdown of a specific gene in *H. tuberculata*. The VP16-N and Eng-N dominant negative system (Kessler 1997) allows us to interfere with normal transcription factor function in both species of *Heliocidaris*.

In this study, we investigate the roles played by *Gsc* and *Msx* in the evolution of direct-development in *H. erythrogramma*. We test the hypothesis that the evolution of direct development in *H. erythrogramma* has proceeded in part through change in expression of a few large effect genes. Misexpression/overexpression and knockdown assays performed in both species as well as *H.e.* × *H.t.* hybrids confirm a vital role in regulated *Gsc* expression for specific features of indirect development. We examine the results in light of the concept of developmental modules and in relation to gene regulatory networks.

MATERIALS AND METHODS

Gsc and *Msx* clones

Full-length cDNA clones of *SpMsx* and *SpGsc* were used for the initial misexpression experiments. Full-length *SpMsx* was a gift from R. Maxson (Dobias et al. 1997), and full-length *SpGsc* was a gift from R. and L. Angerer (Angerer et al. 2001). The *SpMsx* clone was digested with *Xba*I and *Sal*I and transcribed with T7 RNA polymerase using the Ambion (Austin, TX, USA) mMESSAGEmMACHINE kit. The *SpGsc* clone was digested with *Xba*I and transcribed with SP6 RNA polymerase using the same kit. A truncated version of *SpGsc* was produced as an injection control by removal of a 924 bp *Nco*I–*Eco*RV fragment, blunting of the *Nco*I end of the resulting plasmid fragment, and religating of this plasmid. The resulting *SpGscT* clone was then linearized with *Xba*1 and transcribed in the same manner as full-length *SpGsc*. The mRNAs were quantitated by spectroscopy and their integrity checked on denaturing formaldehyde agarose gels before use.

Dominant interference and other construct production

The dominant interference version of *HeGsc* was produced using the VP16-N plasmid, provided by D. Kessler (Kessler 1997). PCR primers of the following sequence were produced: forward 5′-GCCCTCGAG*ATGGAGCAAAAGCTCATTTCTGAAGAGGAC-TTGAATGAAAAG*AGAAAGAGGCGTCATCGT-3′; reverse 5′-GCTCTAGATTA*TACCTTACGCTTCTTCTTTGGAGCCAT*T-CGCTTCTGCTTCCTCCA-3′. The forward primer contains a MYC epitope tag (italicized) and the reverse primer contains a nuclear localization signal (NLS) (italicized) (Li et al. 1999). These primers were used against a *HeGsc* cDNA clone to generate a fragment with the *HeGsc* homeodomain and seven nucleotides of flanking sequence, both a MYC tag and a NLS as well as cut sites for *Xho*I and *Xba*I. The fragment was digested with *Xho*I and *Xba*I, purified and cloned into *Xho*I–*Xba*I digested pBluescript SK+ for forward and reverse sequencing with T3 and T7. Clones containing no sequence mistakes were then removed by restriction digestion and ligated into *Xho*1–*Xba*1 digested pCS2-VP16-N plasmid. Frame and sequence quality were assessed by sequencing with the following sequencing primer made to correspond with the pCS2-VP16-N plasmid: 5′-CAGCCTGGGGGACGAGC-3′.

The *HeGsc* functional mimic was produced using the Eng-N plasmid (provided by D. Kessler). PCR primers of the following sequence were produced: forward 5′-GCCCTCGAGC*ATGGAG-CAAAAGCTCATTTCTGAAGAGGACTTGAATGAAAA*GAGA-AAGAGGCGTCATCGT-3′; reverse 5′-GCTCTAGATTA*TA-CCTTACGCTTCTTCTTTGGAGCCAT*TCGCTTCTGCTTCCT-CCA-3′. The forward and reverse primers again respectively contain a MYC tag and NLS (Li et al. 1999), in addition to an

*Xho*I site on the forward and a *Xba*I site on the reverse. The fragment generated by PCR against *HeGsc* cDNA contains the *Gsc* homeodomain with seven nucleotides of flanking sequence, both a MYC tag and NLS and cut sites for both XhoI and XbaI. The fragment was digested with *Xho*I and *Xba*I, purified, and cloned into pBluescript SK+ for insert sequencing so that a fragment containing no amplification errors could be obtained. The fragment was removed by restriction digestion and cloned into *Xho*I–*Xba*I digested pCS2-Eng-N plasmid. Frame and sequence quality were assessed by sequencing with the following sequencing primer made to correspond with the pCS2-Eng-N plasmid: 5′-CA-CGCCCTCCGCCTTTAC-3′.

Microinjection

All microinjections were carried out using a micromanipulator (Narishige, East Meadow, NY, USA) and a picospritzer (General Valve, Hofheimer, Germany). *H. tuberculata* injections followed Cameron et al. (1996). *H. erythrogramma* and *H. tuberculata* were collected off the coast of Sydney, N.S.W. Australia. Animals were spawned by intracoelomic injection of 0.55 M KCl. *H. tuberculata* eggs were de-jellied for 45 sec in 150 ml sea water brought to a pH of 5 with glacial acetic acid. Acid treatment was stopped by the addition of 14 drops of 1 M Tris pH 8, and the eggs washed with Millipore filtered sea water (MPFSW) (Millipore, Inc., Bedford, MA, USA) three times. Eggs were aligned on a 60 mM Falcon plastic petri dish that had been treated with 1% protamine sulfate for 45 sec, and fertilized under sea water containing 1.6 g/l *p*-amino benzoic acid (PABA). Injections were carried out with constant flow from a glass needle pulled from 5 µl Drummond glass microdispenser tubes (Miller et al. 2002). Needles were broken to form an opening of ≥ 2 µm at the tip. After fertilization, eggs were injected rapidly. It is necessary to remove the needle before the formation of a visible bolus of fluid in the egg. Injection buffer was a solution of 0.12 M KCl and 20% glycerol. Final RNA concentration injected was 200 ng/µl. Injected eggs were washed three times with MPFSW (Millipore, Inc., Bedford, MA, USA), and allowed to develop.

H. erythrogramma eggs were placed along the edge of a 1 mm deep 5–10 mm wide groove made in 60 mm Falcon plastic petri dishes filled with 1% agarose in filtered sea water. The eggs were placed against the wall of the groove on each side, allowing ~ 120 eggs to be quickly injected in a straight line. This agarose had been coated with 0.2% poly-L-lysine for 15 min and washed three times with MPFSW. The same glass needles as used for *H. tuberculata* were used, with a bore sufficient to allow a 20-µm diameter droplet of solution to be ejected with a single pulse under 50 lb/in. of nitrogen pressure. Pressure and needle bore were adjusted throughout the injection of each plate to prevent changes in injection volume due to needle clogging. *H. erythrogramma* were injected with a 40% glycerol and 250–500 ng/µl RNA concentration. After injection, *H. erythrogramma* eggs were removed from the injection dish with a P-1000 pipettor, and placed into MPFSW, fertilized, washed three times with MPFSW, and allowed to develop.

Hybrid injections were carried out as *H. erythrogramma* with the following changes: after injection, eggs were de-jellied in pH 5 sea water for 45 sec–1 min 30 sec, fertilized with *H. tuberculata* sperm, washed three times in MPFSW and allowed to develop.

Tissue morphology and cytology

Embryos were fixed for cytology in 4% paraformaldehyde in MPFSW and dehydrated by ethanol series for storage until use. Embryos were embedded in paraffin as for in situ hybridization (Angerer and Angerer 1991; Kissinger and Raff 1998), then arranged and sectioned in 8-µm serial sections oriented parallel to the animal vegetal axis. Staining was performed with eriochrome cyanin (Chapman 1977).

Immunostaining

Immunostaining for MYC epitope expression was performed on sections fixed in paraformaldehyde and embedded in paraffin. Epitope retrieval was performed on these sections (Taylor et al. 1996; C. R. Smith, personal communication). Briefly, sections were boiled in 0.1 M citric acid, pH 5 in a 1200 W microwave oven for one cycle of 5 min at 80% power, and two more cycles of 5 min at 30% power, replenishing buffer solution between each cycle. Sections were then allowed to cool 30 min at room temperature. Sections were washed 3 × in tris-metabisulfite (TMB) buffer and 0.1% Triton-X 100, blocked in TMB buffer and 10% NGS (Sigma, St. Louis, MO, USA) and incubated overnight at 4 degrees with monoclonal anti-MYC antibody purchased from Zymed/Invitrogen (Carlsbad, CA, USA) at a dilution of 1:500. Sections were washed 3 × in TMB buffer, incubated for 2 h in goat-anti-mouse alkaline phosphatase-conjugated antibody (Zymed) at 1:500, washed 3 × in TMB, 2 × in alkaline phosphatase buffer pH 8, one time in alkaline phosphatase buffer pH 9.5 and stained in alkaline phosphatase buffer pH 9.5 containing 6% low molecular weight polyvinyl alcohol (PVA, Sigma) NBT (4-Nitro blue tetrazolium chloride) and BCIP (5-Bromo-4-chloro-3-indolyl-phosphate) (Boehringer, Indianapolis, IN, USA). Sections were dehydrated and mounted in Permount (Fisher, Pittsburgh, PA, USA).

Microscopy and imaging

Microscopy was performed on Zeiss axioplan microscopes. Photography was by either a 35 mm camera (Zeiss, Gottinger, Germany) and Kodak film (Rochester, NY, USA), or a scope mounted SPOT RT Slider digital camera and captured with SPOT software on a Fryer Co. PC (Fryer Co., Chicago, IL, USA). Images were processed in Adobe Photoshop (San Jose, CA, USA) on a Macintosh computer (Cupertino, CA, USA).

RT-PCR

RNA was isolated by 8 M guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi 1987), and precipitated with 1/10th volume 2 M sodium acetate (NaOAC) and three volumes 100% ethanol, and stored at -80°C. RT-PCR was carried out with the Qiagen One Step kit (Germany) using one half-volume reactions (total reaction volume of 25 µl) and 100 ng of DNase 1-treated total RNA. The following gene specific primers for *Gsc* were used for both *H. erythrogramma* and *H. tuberculata* as well as hybrids: forward 5′-CCGCTGCTTCATCCAGAG-3′; reverse 5′-GTCCATGTCTAACGCTTCC-3′. The competitive RT-PCR fragment for internal standardization was produced by amplification of a *Gsc* fragment from both the *H. erythrogramma* and *H. tuberculata* cDNA clones with the forward primer above as well as a 38-mer designed as described by Celi et al. (1993) with the

following sequence: 5′-GTCCATGTCTAACGCTTCCCACCTC CG-TCATAGCGTCC-3′. This produces a PCR fragment truncated by 49 base pairs from *H. erythrogramma* and 76 base pairs from *H. tuberculata*. After subcloning of the truncated PCR product into the pGEM T easy vector (Promega, Madison, WI, USA) and determining insert direction, sense strand RNA was produced to each truncated gene fragment from the T7 transcription site. RNA was quantified and used for quantitation in the manner described by D. Kaufmann (www.uni-ulm.de/bbartelt/protokoll/rt-pcr.html). The products of the RT-PCR were analyzed by electrophoresis on a 2% MetaPhor agarose gel at 60 V for 6 h, stained in 0.5 μg/ml ethidium bromide for 15 min and de-stained first in tris-borate-EDTA (TBE) buffer (three destaining cycles of 15 min each) and then in distilled water (four cycles of 20 m). Gel photos were digitized on a UMax scanner (Milton Keynes, England) and comparison of bands was made using NIH image software with gel plotting macros. A total of four separate and independent runs were measured for each stage analyzed. The percentage for *H. tuberculata Gsc* was determined by averaging the *H. tuberculata* contribution to total *Gsc* over these four runs. Average deviation from this percentage was determined for each stage. All Gsc PCR fragments obtained were gel purified, subcloned into the pGem T-easy vector and sequenced for confirmation of identity.

RESULTS

Misexpression/overexpressionn of *SpGsc* and *SpMsx* in *H. tuberculata* produces similar phenotypes to those seen in *S. purpuratus*

Misexpression/overexpression, defined here by microinjection of mRNA to over express the gene in all tissues, of *SpMsx* in *H. tuberculata* produces a phenotype identical to that observed in *S. purpuratus* (Tan et al. 1998). Supernumerary spicules are present along with reduced gastrulation (Fig. 1A). Abnormal skeletal patterning continues to be seen in embryos as development proceeds (Fig. 1B). Additionally, a morpholino oligo produced to the 25 bases of sequence 20 bases 5′ to the start codon of *Msx* was used to evaluate the effects of expression knockdown of *HtMsx* in development. In this case, spicule number is normal, but growth is abnormal (Fig. 1, G and H). In addition, gastrulation proceeds abnormally, with no complete archenteron or gut present even when skeletal growth is pronounced (though abnormally patterned) (Fig. 1, I and J). The role of *Msx* in gastrulation and skeletal patterning reported in *S. purpuratus* is retained in *H. tuberculata*.

Misexpression/overexpression assays performed with *SpGsc* in *H. tuberculata* also exhibit a phenotype similar to that reported for *S. purpuratus* (Angerer et al. 2001). There is a failure to properly gastrulate, and embryonic ectoderm adopts a largely oral fate (Fig. 1, C and D) based on the morphology of the ectoderm itself (in this case the ectodermal cells being largely cuboidal). Injection of both an activator construct produced with the *HeGsc* homeodomain linked to a VP16 activation domain as well as injection of a *H.t./H.e. Gsc* morpholino stunts gastrulation and does not allow for the proper differentiation of ectoderm into oral and aboral territories, as no particular cell morphology is predominant (Fig. 1, K–N). These results match well with knockdown of expression of *SpGsc* in *S. purpuratus* by morpholino oligo (Angerer et al. 2001).

The functional role of these two genes appears well conserved between *S. purpuratus* and *H. tuberculata*, two species separated by about 40 million years (Smith 1988). In each case, the differentiation of ectodermal territories as well as gastrulation is disturbed when gene expression is disrupted. *SpMsx* does not appear to affect either skeletogenesis or early gastrulation in *H. erythrogramma*, and the external morphology of the embryos is unchanged (data not shown). *Msx* does have an effect on the amount of mesenchymal tissue as well as proper differentiation of the rudiment (Fig. 2, A and B), indicating profound changes in pattern of expression and function in *H. erythrogramma*. Embryos injected with a truncated version of *SpGsc* used as a control exhibit wild-type development (Fig. 2C).

Misexpression/overexpression of *SpGsc* in *H. erythrogramma*

A crucial hypothesis of this study is that evolutionary changes in expression of transcription factors that are oral ectoderm specific in indirect developers may underlie some of the suite of character changes observed in direct development. Thus, misexpression of such a gene in inappropriate domains of the *H. erythrogramma* embryo could restore some features restored in *H.e.* × *H.t.* hybrids. This hypothesis has been borne out in studies of *Gsc* misexpression/overexpression in *H. erythrogramma.*

We first made observations of the external features of *H. erythrogramma* misexpressing *SpGsc*. *H.e.* × *H.t.* hybrid larvae exhibit a characteristic and highly modified morphology that includes an oral hood and shelf, a mouth, a complete ciliary band surrounding the oral ectoderm, and a lobed overall shape (Raff et al. 1999). *H. erythrogramma* injected with a truncated version of *SpGsc* as a control develop with a wild-type morphology, with an incomplete ciliary band and the characteristic ovoid shape. External morphology reminiscent of the hybrid larvae is produced in $\sim 41\%$ of *H. erythrogramma* embryos injected with full-length *SpGsc* (Fig. 3, C and D). The most obvious change is to the overall shape of the embryo. The characteristic ovoid shape of *H. erythrogramma* gives way to a lobed embryo with a distinct waist near its vegetal end and a curved appearance to the animal vegetal axis. Within this overall change in form there are several smaller and notable morphological details. The blastopore fails to close, leaving an open aboral orifice. The larva forms an oral ciliary shelf, much like that of hybrid

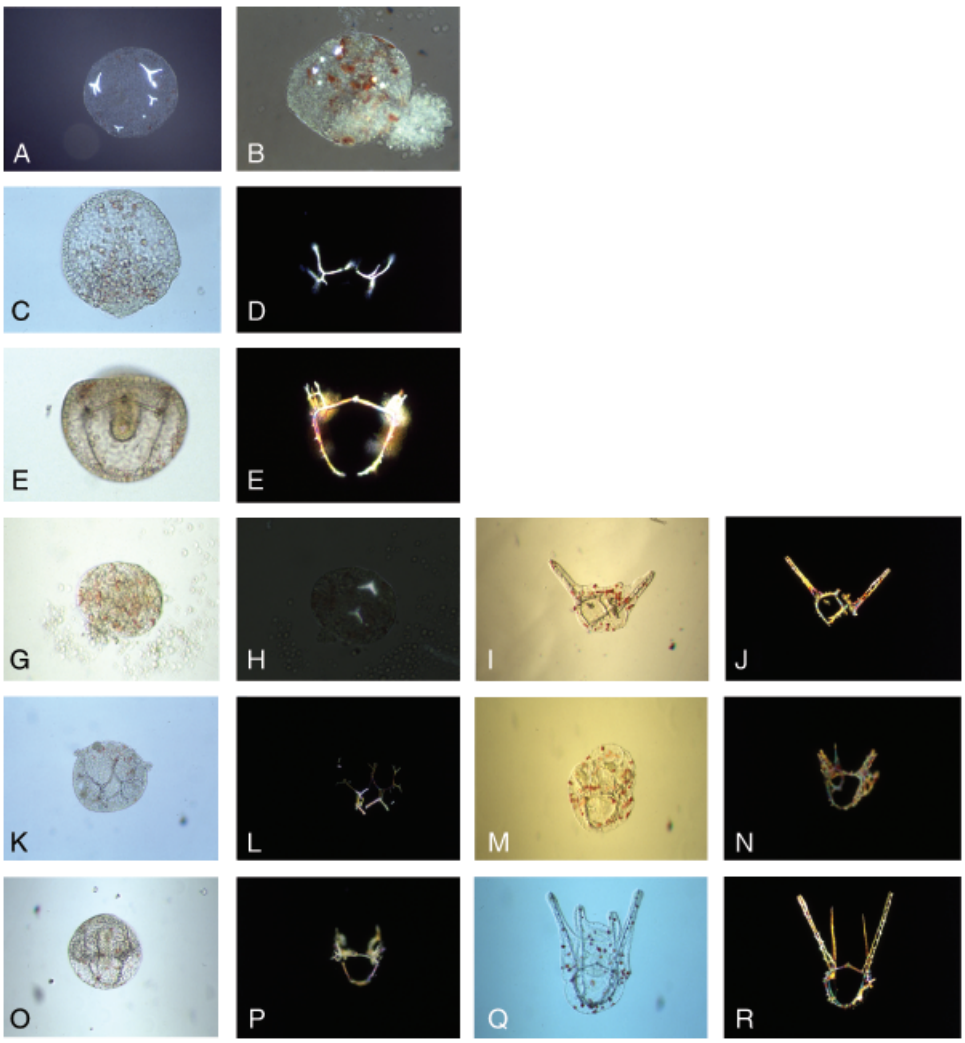

Fig. 1. Results of misexpression/overexpression and knockdown studies of *Msx* and *Gsc* in *Heliocidaris tuberculata*. (A) 24 h *H. tuberculata* injected with full-length *SpMsx* mRNA shows supernumerary spicules, lack of skeletal development, failure of gastrulation, and a uniform ectoderm. (B) 48 h *H. tuberculata* injected with full-length *SpMsx*, exhibits continued failure of skeletal growth from supernumerary spicules, lack of internal structure, and a uniform ectoderm. Embryo has been flattened to better visualize skeleton. (C) 24 h *H. tuberculata* injected with full-length *SpGsc* exhibits nearly uniformly cuboidal ectoderm and failure of gastrulation. (D) Polarized light image of embryo in (C) showing presence of a correct number and relative position of skeletal elements, with arrested skeletal growth. (E) 24 h *H. tuberculata* injected with truncated *SpGSC* and exhibiting wild-type morphology as well as (F) wild-type skeletal pattern and growth. (G) 24 h *H. tuberculata* injected with a morpholino oligonucleotide to *HtMsx* shows undifferentiated ectoderm and no archenteron as well as (H) correct spicule number with no skeletal elaboration. (I) 116 h *H. tuberculata* injected with a morpholino to *HtMsx* and exhibiting abnormal overall morphology including little internal tissue and (J) reduced skeletal growth. (K) 24 h *H. tuberculata* injected with a morpholino to *HtGsc* exhibits undifferentiated ectoderm, failure of gastrulation and (L) improperly branching skeletal elements. (M) 116 h *H. tuberculata* embryo that has been injected with a morpholino to *HtGsc* exhibits distorted overall morphology with a thin, uniform ectoderm, little to no internal structure and (N) reduced skeletal growth. (O & P) 24 h *H. tuberculata* injected with a standard control morpholino exhibit wild-type gastrulation, ectoderm differentiation and normal skeletal pattern. (Q, R) 116 h control morpholino injected *H. tuberculata* show wild-type morphology and skeletal patterning. Whenever possible to determine, animals are oriented oral/animal to the top and aboral/vegetal to the bottom.

embryos. The ciliary band extends in length and outlines an area reminiscent of the hybrid oral ectoderm.

Given the nature of *Gsc* as a repressor of transcription, constructs were built to both mimic and counter the action of *Gsc* domains. These constructs are based on the system of dominant negative plasmids constructed by Kessler (1997), which have been successfully used in sea urchin embryos (Li et al. 1999; Angerer et al. 2001). In order to mimic the function of full-length *Gsc* and eliminate the possibility that the effect we see in *H. erythrogramma* is due to sequence diver-

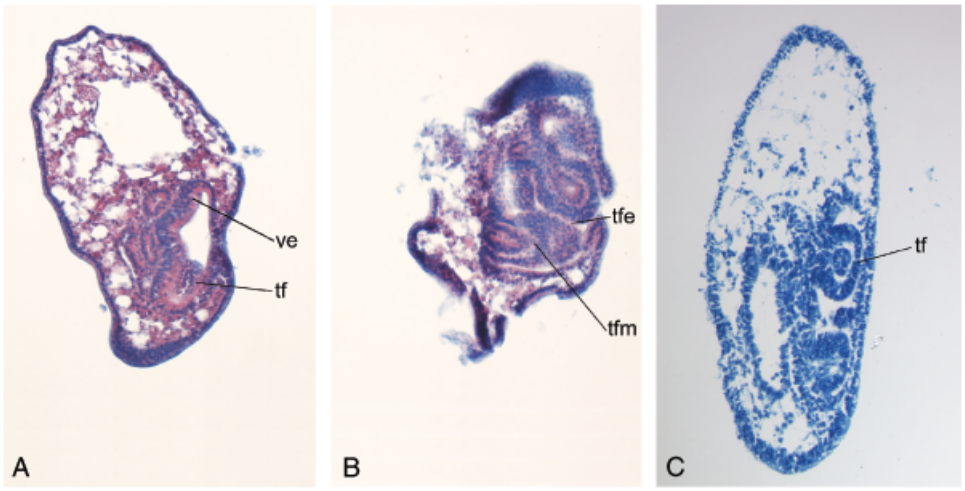

Fig. 2. Effects of *Msx* misexpression/overexpression in *Heliocidaris erythrogramma*. 37 h *H. erythrogramma* embryos injected with full-length *SpMsx* mRNA (A, B) or 45 h *H. erythrogramma* injected with Eng-N control mRNA (C). (A) Longitudinal section with ventral (oral) facing the viewer and animals left side on viewers right. Sections show hypertrophy of tube feet and reduction of coelomic mesoderm. (B) Cross section through plane of rudiment in *SpMsx*- injected *H. erythrogramma*. In embryo B, enlarged tubefeet have formed, but there is little coelomic cavity or accompanying tissues, and hypertrophy of tube foot mesoderm and ectoderm is evident. (C) Animal/vegetal section of 45 h *H. erythrogramma* injected with truncated Eng-N mRNA as a control oriented as A. Normal amounts and patterning of mesodermal and rudiment structures are observed. Abbreviations: ve, vestibule, tf, tube foot.

gence between *S. purpuratus* and *H. erythrogramma Gsc* in the coding region, the Eng-N plasmid (Kessler 1997) was fused to the *H. erythrogramma Gsc* homeodomain. This plasmid incorporates the engrailed repressor domain from *Drosophila* with the target specificity and dimerization capabilities of *H. erythrogramma Gsc*. The injection of this mRNA produces the same skeletal and morphological phenotypes as injection of full-length *SpGsc*.

The striking external morphology of *H. erythrogramma* embryos injected with *SpGsc* and Eng-*HeGsc* fusion mRNA constructs is complimented by equally interesting internal and cellular morphology.

Skeletal development in indirect developing echinometrid sea urchins consists of early onset of both postoral and posterodorsal arms from the initial paired spicules, which in *H. tuberculata* are fenestrated. The presence of two pairs of spicules corresponding to the postoral and posterodorsal spicules in *H. erythrogramma* was documented and examined extensively by Emlet (1995). Growth of extensive larval skeleton is accompanied by the growth of spicules into skeletal plates destined to become adult skeleton, even in fairly early (45 h) larvae (Emlet 1995 and personal observations). This adult skeleton is readily identified by its plate-like appearance as opposed to rod-like arm structures.

H.e. × *H.t.* hybrid embryos produce an extensive larval skeleton that includes long, fenestrated arm rods (Raff et al. 1999). We performed skeletal squashes on *H. erythrogramma* larvae misexpressing *SpGsc* to determine if skeletal growth was being affected. *H. erythrogramma* embryos injected with full-length *SpGsc* rarely initiate adult skeletal growth even in the presence of extensive larval skeletal growth at much later stages (72 h), as shown by a skeletal squash of *H. erythrogramma* injected with truncated *SpGsc*. The skeletal morphology of nearly all (~ 95%) *SpGsc* and *Gsc* functional mimic injected *H. erythrogramma* is similar to the hybrid larval skeleton (Fig. 4, B–D).

In sections parallel to the animal–vegetal axis, vestibular structure is absent, although an archenteron is formed. The embryos have gastrulated, unlike the effect seen by misexpression/overexpression of *Gsc* in indirect developers (Angerer et al. 2001, and this study). The archenteron appears to form neither a well-defined gut as a hybrid, nor the pronounced coelomic pouches of *H. erythrogramma*. There is a mouth-like structure in the oral face, but it does not connect to a gut (data not shown).

Expression of constructs designed to knockdown *Gsc* activity in *Heliocidaris*

The morphology of *H. erythrogramma* injected with full-length *SpGsc*, so highly reminiscent of *H.e.* × *H.t.* hybrid embryos, is a phenotype unlike any previously seen with injection of any other transcription factors into the direct developer (Kauffman and Raff 2003; Nielsen et al. 2003, unpublished data; Zhou et al. 2003). Additionally, fusing the *HeGsc* DNA binding domain to an engrailed repressor domain effectively mimics overexpression of full-length *SpGsc*. To specifically knock down the activity of the *Gsc* gene, we

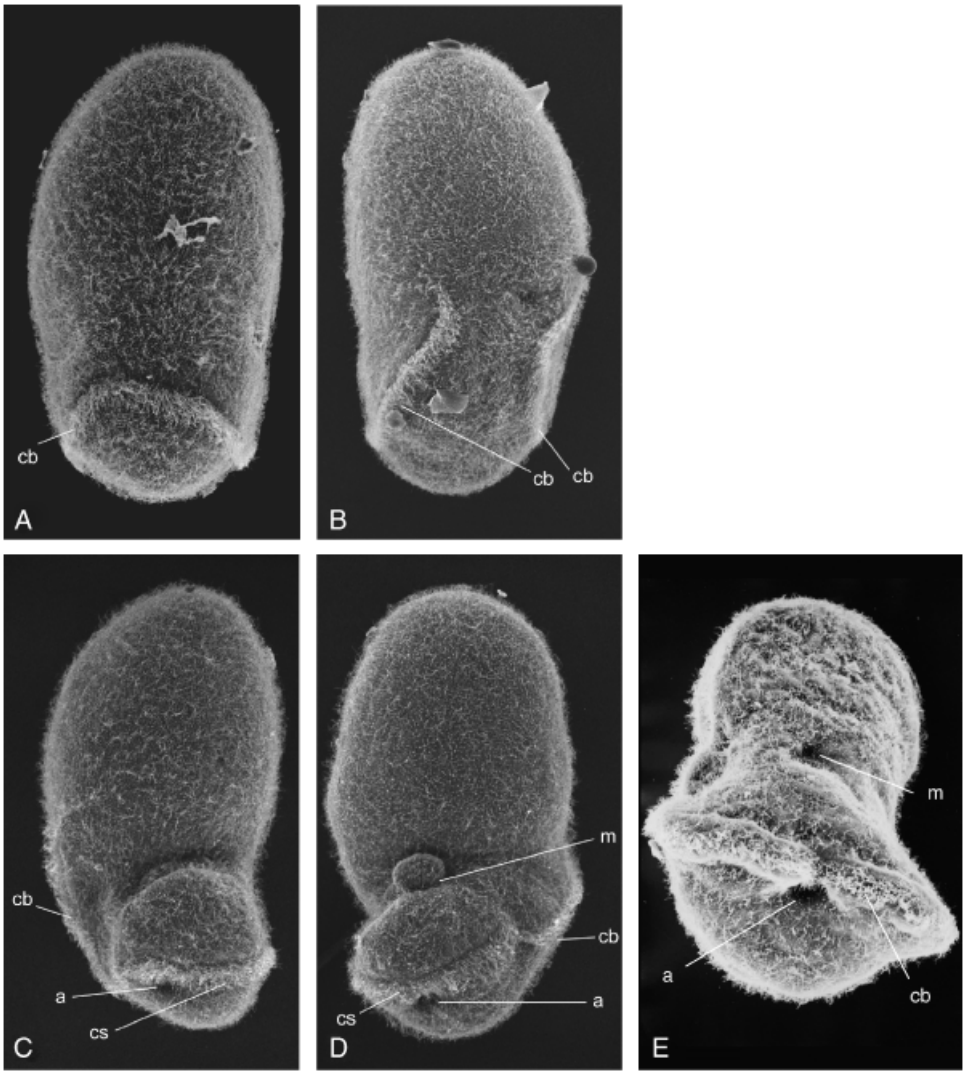

Fig. 3. Effect of *Gsc* misexpression/overexpression in *Heliocidaris erythrogramma*. Scanning electron microscopy images of 48 h embryos. (A) Wild-type *H. erythrogramma* showing normal morphology of ventral (“oral”) face. (B) Dorsal (“aboral”) view of wild-type *H. erythrogramma*. Note ciliary band in two upturned lines. (C) Ventral view of *H. erythrogramma* injected with *SpGsc* mRNA; mouth region is poorly formed. (D) Ventral view of a second *H. erythrogramma* injected with *SpGsc* mRNA. A mouth is present (a tissue bleb protrudes from the mouth opening). (E) Ventral (oral) view of a 2-day-old *H.e.* × *H.t.* hybrid. Mouth, ciliary band, and anus are all indicated (reproduced from Raff et al., 1999). All embryos are oriented animal top and vegetal bottom. Abbreviations: cb, ciliary band; m, mouth; a, anus; cs, ciliary shelf.

have designed a construct that should both compete and interfere with endogenous *HeGsc*. This construct is also based on Kessler’s (1997) system of transcription factor dominant negative interference. Because GSC is known to function as a transcriptional repressor in a number of systems including echinoids (Isaacs et al. 1999; Li et al. 1999; Takeda et al. 2000), coupling the *Gsc* homeodomain to a strong transcriptional activator should accomplish three things: (1) because GSC functions as a dimer whose pairing ability is contained within the homeodomain of the protein, the addition of exogenous copies of this homeodomain should dimerize with endogenous transcript and compete in dimerization, (2) the exogenous GSC should compete with GSC for target sites, reducing the amount of endogenous GSC bound, and (3) the strong activator coupled to the *Gsc* homeodomain should activate targets which are supposed to be repressed by *Gsc* or interfere with the ability of the endogenous protein to repress transcription when dimerized with this exogenous transcript. The injection of a VP16-*Gsc* construct mimics the effect seen by *Gsc* morpholino injection in *H. tuberculata*, causing a failure of both gastrulation and ectoderm differentiation. The knockdown effect of VP16-*Gsc* is comparable to the effect of morpholino oligo knockdown in both *S. purpuratus* and *H. tuberculata*, and should therefore be an effective construct for investigating the knockout of *Gsc* in *H. erythrogramma*.

The external anatomy of *H. erythrogramma* injected with VP16-*Gsc* indicates that the transformation of *Gsc* into an activator has little to no effect on the early development of the larva, and normal morphology and timing of development of adult skeleton indicate no dramatic change in rudiment development. No effect in gastrulation could be seen in section. Given this lack of effect for *Gsc* knockdown, sections of VP16-*HeGsc* as well as VP16-N control injected

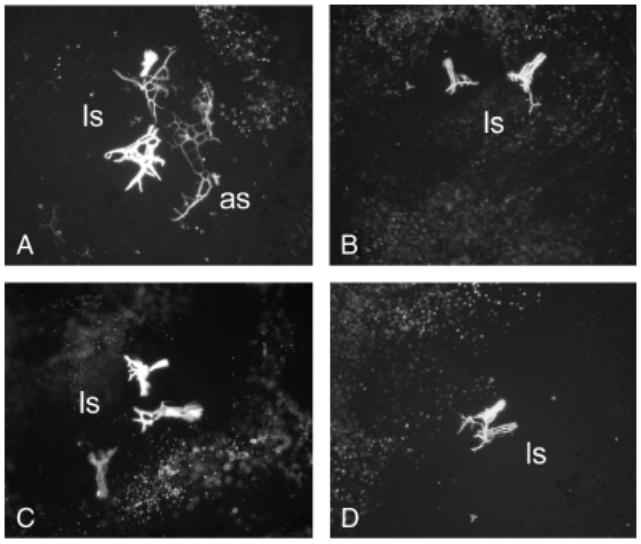

Fig. 4. Effect of *Gsc* on skeletal development in *Heliocidaris erythrogramma*. Polarized light image of skeletal squashes of full-length and truncated *SpGsc* mRNA-injected *H. erythrogramma* and *H.e.* × *H.t.* hybrid embryos. (A) 63 h truncation control (*SpGscT*) injection in *H. erythrogramma* showing extensive adult skeletal plate growth. (B–D) 63 h *H. erythrogramma* injected with *SpGsc* mRNA showing a marked lack of adult skeletal growth and extensive larval skeletal growth. Abbreviations: as, adult skeleton; ls, larval skeleton.

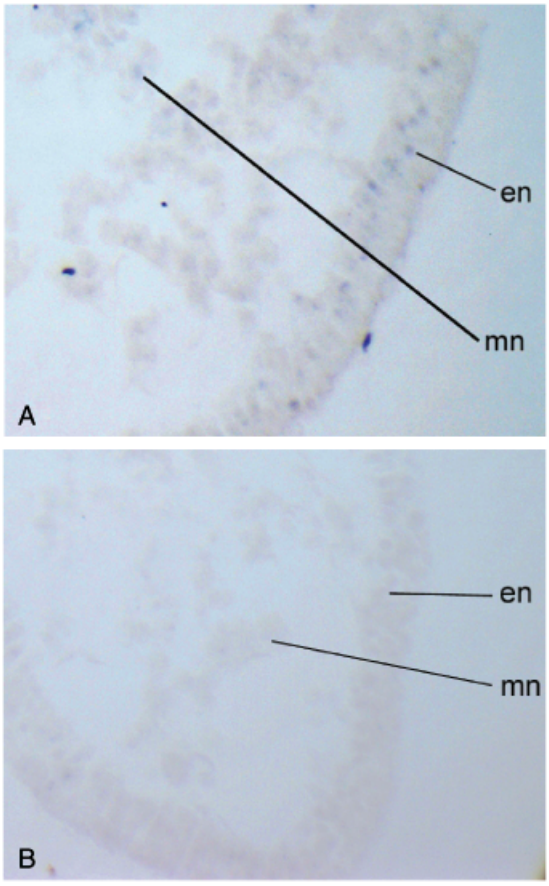

Fig. 5. Confirmation of protein expression in VP16-*HeGsc*-injected embryos. (A) Section through *Heliocidaris erythrogramma* injected with VP16-*HeGsc* and stained for expression of C-Myc. Note nuclear staining in ectoderm and mesenchyme. (B) Section through *H. erythrogramma* injected with VP16-N mRNA and stained for expression of C-Myc. Abbreviations: en, ectodermal nucleus; mn, mesenchymal nucleus.

H. erythrogramma were prepared and stained for the presence of a MYC epitope incorporated into the VP16-*HeGsc* construct (Fig. 5). Positive nuclear signal indicates not only presence of protein product from this construct, but proper nuclear localization of the product. It is apparent that activation interference with *Gsc* does not perturb gross aspects of *H. erythrogramma* development as overexpression of either full-length *SpGsc* or the Eng-*HeGsc* mimic does.

Expression of a construct designed to knockdown *Gsc* activity in *H.e.* × *H.t.* hybrids

We have shown that *Gsc* expression is restored in the oral ectoderm of *H.e.* × *H.t.* hybrids. In addition, the morphology of *H. erythrogramma* embryos injected with *Gsc* mRNA are modified in the direction of the hybrid morphology. These observations suggest that blocking *Gsc* expression in hybrids should have the effect of reducing hybrid morphology. Both misexpression/overexpression and dominant negative injections were performed on *H.e.* × *H.t.* hybrids. Neither overexpression of *Gsc* nor expression of a dominant negative construct had an overt effect on outward hybrid morphology. Internally, however, the effects of both were clearly discernible. The growth of larval skeletal elements is perturbed in each case, with a slight and temporary delay in skeletogenesis apparent in *SpGsc* misexpression/overexpression studies (Fig. 6, A and B). Injection of the VP16-*HeGsc* knockdown construct causes an irreversible stunting of skeletal growth (Fig. 6, C and E). This effect is what the hypothesis of a role for the evolutionary loss of oral *Gsc* expression in *H. erythrogramma* suggests, and the reduction in skeletal growth observed coincides with a reduction of the differentiated oral ectoderm territory needed to continue this growth.

Control of paternal *Gsc* gene expression in hybrids

We have shown that both the paternal and maternal genomes are expressed in the *H.e.* × *H.t.* hybrid embryos (Nielsen et al. 2000). Additionally, *Gsc* is spatially expressed in the *H. tuberculata* pattern in *H.e.* × *H.t.* hybrids (Wilson et al. 2005). Here we ask if maternally and paternally derived *Gsc* genes are differentially regulated in *H.e.* × *H.t.* hybrids. The experiment was performed with competitive RT-PCR as described previously using primers spanning an area of *Gsc* where

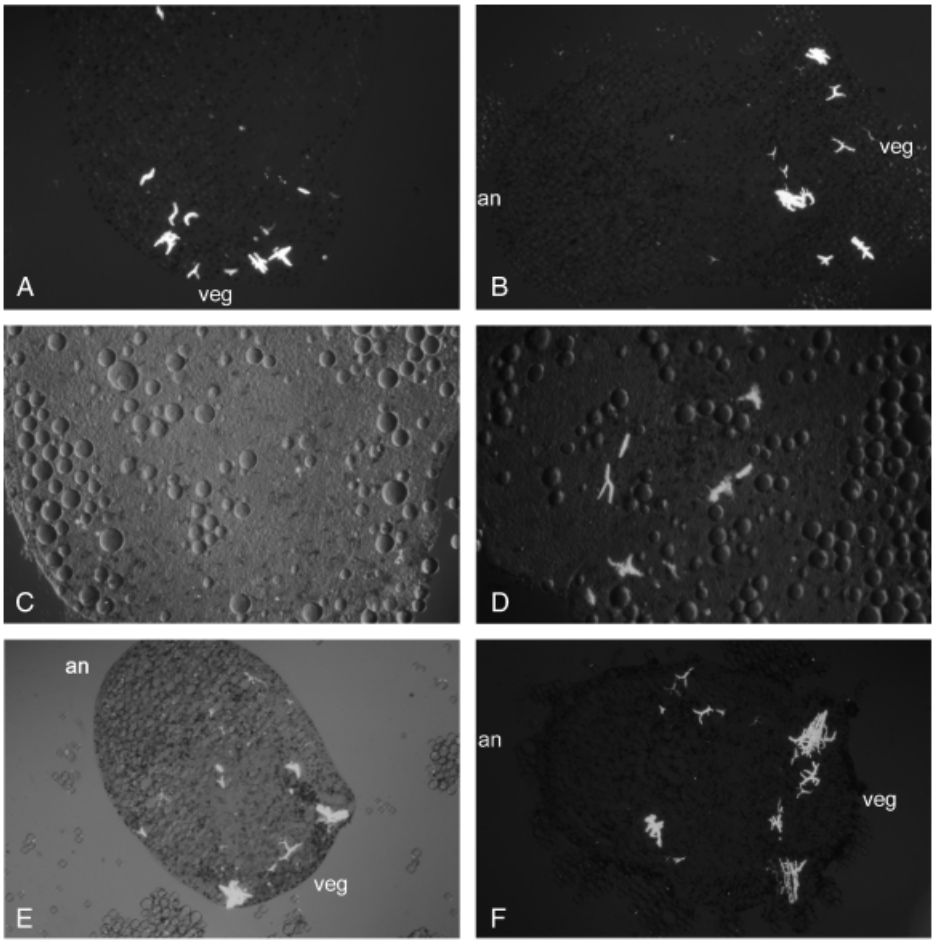

Fig. 6. Effect of misexpression and dominant negative interference with *Gsc* in *H.e.* × *H.t.* hybrid embryos. Embryos were flattened to reveal skelton in polarized light. (A) 60-h hybrid embryo injected with full-length *SpGsc* (× 20 objective). (B) 60-h hybrid embryo injected with truncated *SpGsc* (× 20 objective). (C) 48-h hybrid embryo, vegetal end magnified at × 40, injected with VP16-*HeGsc* and showing little spiculogenesis. (D) More extensive spiculogenesis seen in 48 h VP16-N-injected control hybrid embryos. (E) 62-h hybrid embryo injected with VP16-*HeGsc* construct, illustrating stunted skeletal growth. (F) 62-h hybrid embryo injected with VP16-N control construct with more extensive skeletogenesis. Abbreviations: an, animal; veg, vegetal.

H. erythrogramma is missing 30 bp when compared to the *H. tuberculata* (Wilson et al. 2005), a size readily distinguishable on a 2% MetaPhor agarose gel. Using an amount of competitor determined to produce a roughly equal amount of product as endogenous, 20 cycles of RT-PCR were run with five stages of hybrid RNA for quantification. The two bands were measured from this gel as well as from previous experiments (Wilson et al. 2005), and the ratio of *H. erythrogramma* to *H. tuberculata Gsc* present was determined using NIH Image and graphed with Microsoft Exel (Fig. 7). In the first stage examined (128 cell), the predominant form of *Gsc* expressed is that of *H. erythrogramma*, which is not surprising, as the embryo would still be using maternal mRNA at this stage. By 24 h (gastrula), 16% of *Gsc* expression derives from the *H. tuberculata* genome. By 48 h, 72% of the *Gsc* present is the *H. tuberculata* version, by three days, 83% of the expression is from the paternal genome and finally at 4 days, 81%. The preferential use of the paternal version of *Gsc* in the hybrids demonstrates the dominance of the genome of the indirect developer in the hybrid, as well as reinforcing the probable importance of *Gsc* regulation in the hybrid morphology.

DISCUSSION

Gsc and *Msx* functions are conserved by *H. tuberculata* and other indirect developers

Heliocidaris and *Strongylocentrotus*, which both have pluteus larvae, belong to different families and are approximately 40 million years diverged (Smith 1988). *H. tuberculata* injected with either *SpMsx* or *SpGsc* show an interference with gastrulation and ectodermal differentiation, as in *S. purpuratus* (Tan et al. 1998; Angerer et al. 2001). In addition, *H. tuberculata* injected with the *SpMsx* mRNA show the skeletal mispatterning observed in *S. purpuratus*. *H. tuberculata* injected with full-length *SpGsc* mRNA convert most ectoderm to an oral fate as seen in *S. purpuratus* misexpression/overexpression experiments. Knockout of *HtGsc* by morpholino oligo as well as misexpression/overexpression of the dominant negative VP16-*HeGsc* mRNA duplicate the nondifferentiated ectoderm and failure of gastrulation as seen in *S. purpuratus* (Angerer et al. 2001). Although not performed in *S. purpuratus* to date, the result of gene knockdown on *HtMsx* by morpholino oligo injection as expected stunts skeletal

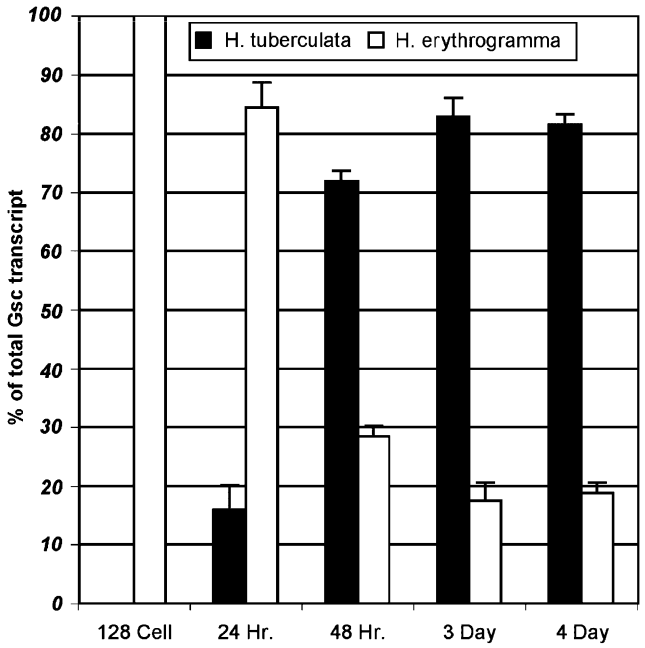

Fig. 7. RT-PCR reveals the temporally progressive dominance of *Heliocidaris tuberculata Gsc*. (A) 128 cell hybrid (B) 24-h hybrid (C) 48-h hybrid (D) 72-h hybrid (E) 4-day hybrid. Percentage given in text is taken from the average measurement of the contribution of *H. tuberculata* to total endogenous product (*H. erythrogramma* +*H. tuberculata*). Error bars indicate average deviation of results from this percentage. Competitor was included to reduce total amplification of each stage and facilitate measurement.

growth and prevents proper gastrulation and ectodermal differentiation. These results indicate a strong conservation of developmental regulatory gene action in indirect-developing sea urchins, consistent with the regulatory conservation shown for indirect developers by other means (Raff et al. 2003).

H. erythrogramma utilizes these genes differently

The role of *Msx* in gastrulation has been reduced in *H. erythrogramma*, as an archenteron forms unlike indirect developers injected with *SpMsx*. The patterning of skeleton as evidenced by visualization in polarized light appears normal (data not shown). This result suggests that patterning of the *H. erythrogramma* skeleton may be de-coupled from ectodermal patterning with the loss of the oral ectoderm module. However, the internal mesodermal structures of *H. erythrogramma* are clearly changed in amount (tube foot mesoderm elevated, coelomic mesoderm reduced) in response to overexpression of *SpMsx*. The important thing about this result is that two transcription factors that play major conserved roles in the pluteus oral ectoderm in *H. erythrogramma* have assumed different domains of function.

In *H. erythrogramma*, mesoderm differentiation is highly accelerated and represents an important heterochrony (Ferkowicz and Raff 2001). The effects of *Msx* misexpression/overexpression on mesodermal development in *H. erythrogramma* may reflect a heterochronic shift in *Msx* function to much earlier than occurs in indirect development. The changes in mesodermal tissues, and hence the mispatterning of the adult animal, is an effect we could not hope to see by misexpression/overexpression studies within an indirect developer, because these events occur weeks after fertilization. The evolution of *Msx* function in *H. erythrogramma* includes a loss of early functions associated with oral ectoderm and skeletogenic mesenchyme patterning, and a hypothesized acceleration of *Msx* function in coelomic mesoderm development.

Gsc has proved to be the most informative gene in its potential role in the evolution of larval form. There is a morphological transformation of injected *H. erythrogramma* towards a hybrid like larva, with a correctly positioned anus, an oral shelf, an apparent oral field, the suppression of rudiment formation and in some cases an apparent mouth. Extravestibular ectoderm in wild-type and control injected *H. erythrogramma* consists of a single columnar layer of cells with basally located nuclei and appears homogenous in section. Two different types of ectoderm are formed in *H. erythrogramma* injected with *SpGsc* and Eng-*HeGsc*. The area identified as the relic oral face of injected *H. erythrogramma* is populated by a thickened cellular morphology and has clear, definite boundaries, although the area does not appear to be surrounded by a complete ciliary band. The rest of the ectoderm is characteristic of *H. erythrogramma*, with basally located nuclei within a single layer of columnar epithelium. The skeletal structure of *SpGsc* and Eng-*HeGsc* injected *H. erythrogramma* resembles that of an indirect developer, although no larval arms are ever seen to develop. Growth of adult skeletal plates is never observed in these embryos.

We asked if there was some difference in the *H. erythrogramma* and *H. tuberculata* GSC proteins that can account for the changes seen in *H. erythrogramma* morphology. There are two domains present in *Gsc* conferring its activity, both of which are intact not only in *S. purpuratus*, but also in both *Heliocidaris* species. *Gsc* belongs to the paired class of homeodomain proteins, and thus its target site specificity relies in part on formation of cooperative homodimers whose targets consist of palindromic repeats of 5′-TAAT-3′ separated by a variable number of nucleotides (Tucker and Wisdom 1999). Amino acid position 50 in the paired class of homeodomains confers the next level of binding specificity to the transcription factor. The presence of a lysine at position 50, confers the binding preference of GSC to a P3C binding site (5′-TAATCCGATTA-3′) (Goriely et al. 1996; Tucker and Wisdom 1999), and is present in all three echinoid species examined, as well as all organisms from which the gene has been cloned. The conservation of seven amino acids comprising the GEH (Goosecoid Engrailed Homology) domain

(Goriely et al. 1996) located near the N-terminus of the protein is also notable (Wilson et al. 2005). This region presumably confers at least part of the repression properties to this protein as well as that of engrailed itself (Goriely et al. 1996; Mailhos et al. 1998). Both the homeodomain and the GEH are highly conserved across phyla, and define the homology of goosecoid. Thus, the functional properties of GSC as a transcriptional repressor binding P3C regulatory elements, based on these regions identified as crucial for its function across a wide range of metazoans, are present in both *Heliocidaris* species.

Although there is extensive modification of the *H. erythrogramma* larva with the misexpression/overexpression of both *SpGsc* as well as the mimic construct Eng-*HeGsc*, there appears to be no effect on the larva with the misexpression/overexpression of the VP16-*HeGsc* construct. Gastrulation, adult skeletal formation and overall morphology of *H. erythrogramma* are unaffected by interference with *Gsc*, implying a diminished role (or at least sensitivity to reduction) for *Gsc* in the earliest stages of development within the direct developer when compared to the indirect developers. This is in contrast to the phenotypes observed in both *H. tuberculata* and *S. purpuratus* with both morpholino oligonucleotides and dominant negative misexpression (Angerer et al. 2001, this study).

Changes in *Gsc* action are due to regulatory evolution

The morphology produced by injection of *SpGsc* and Eng-*HeGsc* mRNAs into *H. erythrogramma*, although striking, is not that of a fully indirect developing embryo. However, it is also clear that it represents a significant effect for expression of a single gene.

RT-PCR shows that *H. tuberculata* and *H. erythrogramma* *Gsc* transcription is differentially regulated in the hybrids, indicating differences in transcriptional regulatory sites and the existence of a dominating *H. tuberculata* upstream regulatory network. The temporally progressive dominance of *H. tuberculata* *Gsc* in the hybrids indicates the importance of this upstream regulation, as the known functional regions of the *Gsc* genes of both species are identical. Although we were already aware of the presence of paternal transcripts in the hybrids, this preferential utilization of a specific and powerful transcription factor from the paternal genome was unexpected, as previous studies have shown a preferential down playing of the paternal genome (Tufaro and Brandhorst 1982). Additionally, *Gsc* expression is localized to the oral ectoderm of the hybrid embryos (Wilson et al. 2005). This localization is different than that for a later stage *H. erythrogramma* and indicates differential spatial regulation of *Gsc* expression in the hybrid context, which also indicates that other important components specific to the developmental phenotype resulting from the changed regulation of *Gsc* in *H. erythrogramma* lie upstream of *Gsc*. At least one gene, *Spdri*, has now been shown to be cross-regulatory with *Gsc* expression in the indirect-developing sea urchin *S. purpuratus* (Amore et al. 2003). Further, Duboc et al. (2004) have shown that *Gsc* and other genes crucial to the establishment of the oral–aboral axis and oral ectoderm differentiation are regulated by *Nodal*, providing an indication of the mechanisms for upstream regulation of *Gsc* in patterning the indirect-developing sea urchin embryo oral–aboral axis. The differential effect in expression of parental *Gsc*'s observed in hybrids suggest that control of the *Gsc*'s of *H. tuberculata* and *H. erythrogramma* differ, and that the *H. tuberculata* system comes to prevail in the hybrids.

Evolution by modularity

Large evolutionary changes proceeding by action of a few genes has been proposed as important in other systems (Rieseberg and Ellstrand 1993; White and Doebley 1998; Lukens and Doebley 2001) and seems to be at work within sea urchins as well. A demonstration of large evolutionary changes happening rapidly followed by small changes has been reviewed (Lenski and Travisano 1994; Leroi 2000), and seems appropriate in this instance to explain the role of *Gsc* in the evolution of direct development. The long-term integration of *Gsc* into a genetic module represented by the oral ectoderm of the pluteus disintegrated over an evolutionarily relatively short term (approximately four million years) (Zigler et al. 2003).

The loss of the oral ectoderm module in *H. erythrogramma* did not necessitate the loss of either *Msx* or *Gsc*. Both *Msx* and *Gsc* have undergone significant evolutionary changes in both the site and timing of their expression. Functionally, however, the two genes have evolved in different ways. *Msx* has lost all early aspects of oral localization in *H. erythrogramma*. In addition, the downstream elements controlling larval skeletal patterning that respond to *Msx* in both *H. tuberculata* and *S. purpuratus* are no longer able to respond to *Msx* misexpression/overexpression in *H. erythrogramma*. *Msx* has been dissociated from the oral module altogether.

We hypothesize that *Gsc* has been lost from a component of oral ectoderm developmental control causing its expression to fade early in the oral region. The transience of *Gsc* expression may have been crucial to the loss of oral ectoderm, although later elements of the differentiation of the oral module have remained competent to respond to *Gsc*. Thus, the first phase of differentiation of the oral ectoderm module may still occur in *H. erythrogramma*, as evidenced by the early expression of *Gsc* in the presumptive "oral" field and the later morphological expression of the partial ciliary band. The transformation of the *H. erythrogramma* "oral" field into a more definitive oral ectoderm in embryos injected with *Gsc* mRNA may result from the persistent expression of *Gsc* in the competent region. The persistence of *Gsc* in the oral ectoderm

domain of *H.e.* × *H.t.* hybrids may be governed by the dominant *H. tuberculata* upstream regulation of *Gsc* expression evident in the hybrids.

There is one more interesting observation that should be addressed. Knockdown of *Gsc* by injection of VP16-*HeGsc* mRNA into *H.e.* × *H.t.* hybrid embryos does not affect ectoderm. Our interpretation of this result is that we are seeing a graded effect with a resulting incomplete knockdown of *Gsc* coupled with an upregulation of oral ectoderm specific *H. tuberculata Gsc*. The oral ectoderm likely has sufficient *Gsc* to differentiate. The skeleton is affected, probably indicating that some function of hybrid oral ectoderm is insufficient to support skeletal growth in the VP16-*HeGsc* injected embryos.

A brief evolutionary scenario

We can begin to suggest an approximate ordering of events in the evolution of the *H. erythrogramma* larva that took place in less than the four million years separating *H. erythrogramma* from *H. tuberculata* (Zigler et al. 2003). The first step was likely a change in oogenesis resulting in a large egg and release from the need for larval feeding (Wray 1996; Byrne et al. 1999). This would have resulted in a large facultative-feeding pluteus like that known for *Clypeaster rosaceus* (Emlet 1986). Such forms have sufficient maternal stores not to have to feed, freeing internal tissues for changes promoting more rapid development of adult features, including the morphological and molecular heterochronies seen in development of the rudiment (Ferkowicz and Raff 2001). There are several plausible representatives of a postulated second "step" in known intermediate forms between feeding plutei and forms like *H. erythrogramma* (Wray 1996). These intermediate forms retain larval arms and an apparent oral ectoderm, but lack functional guts. We suggest that the third "step" in the evolution of *H. erythrogramma* may have involved a change in *Gsc* expression (and possibly other genes) that would have produced a rapid loss of oral ectoderm and related ectodermal structures. Fourth, co-option of genes from the oral ectoderm into new functions (or elimination of their larval function) would have taken place. Finally, gains of maternal regulation of embryonic axes would have occurred, possibly concurrently with the loss of oral expression of *Gsc,* and co-option of its primary role to the left side vestibular ectoderm (Wilson et al. 2005). These series of events would have required relatively few gene changes in the initial events of functional losses, with a likely slower train of subsequent regulatory changes to fine tune direct development (Leroi 2000).

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