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T-brain homologue (*HpTb*) is involved in the archenteron induction signals of micromere descendant cells in the sea urchin embryo

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SUMMARY

Signals from micromere descendants play a crucial role in sea urchin development. In this study, we demonstrate that these micromere descendants express *HpTb*, a *T-brain* homolog of *Hemicentrotus pulcherrimus*. *HpTb* is expressed transiently from the hatched blastula stage through the mesenchyme blastula stage to the gastrula stage. By a combination of embryo microsurgery and antisense morpholino experiments, we show that *HpTb* is involved in the production of archenteron induction signals. However, *HpTb* is not involved in the production of signals responsible for the specification of secondary mesenchyme cells, the initial specification of primary mesenchyme cells, or the specification of endoderm. *HpTb* expression is controlled by nuclear localization of β-catenin, suggesting that *HpTb* is in a downstream component of the Wnt signaling cascade. We also propose the possibility that *HpTb* is involved in the cascade responsible for the production of signals required for the spicule formation as well as signals from the vegetal hemisphere required for the differentiation of aboral ectoderm.

Key words: Archenteron induction signal, T-brain, Sea urchin

INTRODUCTION

In the sea urchin, the fourth cleavage produces a 16-cell stage embryo with eight mesomeres in the animal hemisphere and four macromeres and four micromeres in the vegetal hemisphere. Micromeres are specified autonomously, as isolated micromeres give rise to skeletogenic cells in vitro (Okazaki, 1975), and no other fate is ever observed for micromeres when transplanted to ectopic location in the embryo. In addition, micromeres play an important role in axis formation, as shown by deletion of micromeres or their transplantation of micromeres into the animal pole region (Hörstadius, 1973; Ransick and Davidson, 1993). Removal of micromeres during the period from fourth and fifth cleavage also impairs expression of an endoderm specific gene (Ransick and Davidson, 1995). Furthermore, signal(s) emanating from micromere descendants at late blastula stages are important for gastrulation itself (Minokawa and Amemiya, 1999; Ishizuka et al., 2001).

Recently, progress has been made in identifying molecular mechanisms that underlie the specification and subsequent differentiation of the micromere-primary mesenchyme cell (PMC) lineage (reviewed by Davidson et al., 1998; Angerer and Angerer, 2000). First, genes encoding proteins involved in the formation of spicule have been identified, including *SM50* (Benson et al., 1987) and *SM30* (George et al., 1991). The *cis*-regulatory systems controlling the expression of *SM50* (Makabe et al., 1995) and *SM30* (Akasaka et al., 1994; Frudakis and Wilt, 1995; Yamasu and Wilt, 1999) were analysed in detail. One of the transcription factors responsible for *SM50* and *SM30* expression is Ets; *HpEts* induces the expression of *HpSM50* and loss of *HpEts* function results in the failure of spicule formation (Kurokawa et al., 1999). Second, nuclear localization of β-catenin is essential for the autonomous specification of micromere (Wikramanayake et al., 1998; Logan et al., 1999; Emily-Fenouil et al., 1998). Third, Delta, which is expressed by micromere descendants, plays an essential role in the Notch-dependent specification of SMCs (Sweet et al., 1999; McClay et al., 2000; Sherwood et al., 2001; Sweet et al., 2002).

It has been demonstrated that transcription factors containing a T-domain, the DNA-binding domain homologous to the mouse *brachyury* (or *T*) gene product, play important roles in various aspects of animal development (reviewed by Herrmann and Kispert, 1994; Smith, 1997; Papaioannou and Silver, 1998). T-domains fall into a number of subfamilies,

such as *brachyury*, *Tbx* and *T-brain*. *T-brain-1*, which is expressed in the cerebral cortex (leading to the name T-brain) was first isolated from mouse (Bulfone et al., 1995). A related T-box gene, referred to as *Eomesodermin,* isolated from *Xenopus laevis* is first expressed in the mesoderm and then expressed in the most anterior part of the brain at the tadpole stage (Ryan et al., 1996; Ryan et al., 1998). Recently, invertebrate homologues of *T-Brain-1* have been isolated from a hemichordate acorn worm (Tagawa et al., 2000), a starfish (Shoguchi et al., 2000) and a sea cucumber (Maruyama, 2000).

We report the isolation and characterization of sea urchin homologue of *T-brain-1*, referred to as *HpTb*. We suggest that *HpTb* is involved in the production of signals from micromere progeny responsible for gastrulation. We also propose that *HpTb* is involved in the cascade responsible for the production of signals required for the spicule formation, and for signals from the vegetal hemisphere required for the differentiation of oral-aboral ectoderm.

MATERIALS AND METHODS

Embryo culture

Gametes of the sea urchin (*H. pulcherrimus*) were obtained by coelomic injection of 0.55 M KCl, and fertilized eggs were cultured in the artificial sea water Jamarin U (Jamarin Lab) at 16°C.

Cloning of cDNA for the sea urchin homologue of the mouse *T* gene

The amino acid sequences of T domain of the *T* gene products are highly conserved among mouse (Herrmann et al., 1990), *Xenopus* (Smith et al., 1991), zebrafish (Schulte-Merker et al., 1992), ascidians (Yasuo and Satoh, 1994) and sea urchins (Harada et al., 1995). The sense-strand oligonucleotide that corresponds to the amino acid sequence YIHPDSP and the antisense oligonucleotide that corresponds to the amino acid sequence NPFAKG(A)L(F) were synthesized using an automated DNA synthesizer (Applied Biosystems). Using these oligonucleotides as primers, we amplified target fragments from an *H. pulcherrimus* gastrula cDNA library by means of PCR. Probing with candidate cDNA fragments random-labelled with [^{32}P]-dCTP (Amersham), we screened the library at high stringency (hybridization, 6×SSPE, 0.1% SDS, 1×Denhardt's solution, 50% formamide at 42°C; washing, 2×SSC, 0.1% SDS at 65°C). The isolated clones were subcloned into pBluescriptII SK(+) (Stratagene). The clones were sequenced by dideoxy chain termination (Sanger et al., 1992). In order to obtain a cDNA clone that contains entire open reading frame, we re-screened *H. pulcherrimus* hatched blastula cDNA library with an RNA probe synthesized from the obtained cDNA. The RNA probe was labelled with digoxigenin (DIG)-11-UTP (Roche) using T3 Megascript kit (Ambion) as described in the instruction manual. An antibody against digoxigenin that had been conjugated to alkaline phosphatase was used to probe the membrane (Roche). The chemiluminescent signal produced by enzymatic dephosphorylation of CSPD (TROPIX) by alkaline phosphatase was detected by X-ray film.

Northern blot hybridization

The RNA was extracted from *H. pulcherrimus* embryos at various developmental stages as described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). The total RNA (2 μg) was electrophoresed on each lane of a denaturing formaldehyde-1% agarose gel, transferred to a Nytran membrane (Schleicher and Schuell), and hybridized to the antisense RNA of *HpTb* labelled with DIG-11-UTP. The signal was detected as described above.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described previously (Kurokawa et al., 1999). DIG-labelled antisense RNA probe was prepared with an Ambion's Megascript kit using DIG-11-UTP. Riboprobes were hydrolyzed with alkali to sizes of about 150-400 nucleotides, as described by Cox et al. (Cox et al., 1984).

Synthetic mRNA and antisense morpholino microinjection into sea urchin eggs

To generate DNA templates for in vitro RNA synthesis, the plasmids that contain cDNA for HpTb, truncated HpEts-FLAG (Kurokawa et al., 1999) and the intracellular domain of sea urchin LvG-cadherin (Logan et al., 1999) were linearized with restriction enzymes. 5′ capped mRNA was synthesized by using the T7 Megascript kit (Ambion) and Cap Analog [m7G(5′)ppp(5′)G; Ambion] as described in the instruction manual. Microinjection of sea urchin eggs was done as described by Gan et al. (Gan et al., 1990). Morpholino oligonucleotides complementary to sequence containing the translation start site of HpTb AAATTCTTCTCCCATCATGTCTCCT and the control *lacZ* morpholino were obtained from Gene Tools (Corvallis). Oligonucleotides were dissolved in 40% glycerol at a concentration of 5 pg/pl (3.5×10^8 molecules/pl). Two picolitres of the solution was injected into each fertilized egg.

Indirect immunostaining

The cDNA fragment coding for the N-terminal region of HpEts protein, corresponding to codons 450 bp to 881 bp, and the N-terminal region of HpTb protein, corresponding to codons 291 bp to 707 bp and 807 bp to 1373 bp, were fused downstream to the *malE* gene in the pMAL-cRI vector (New England Biolabs), which encodes the *E. coli* maltose-binding protein (MBP). The fusion proteins, HpEts-MBP and HpTb-MBP, were produced in *E. coli*, affinity purified using an amylose resin, and used for immunization of rabbits to generate anti-HpEts and anti-HpTb, respectively.

Antibodies were purified using affinity column containing specific antigens (Harlow and Lane, 1988). Embryos were fixed and stained with affinity-purified anti-HpEts polyclonal sera or affinity-purified anti-HpTb polyclonal sera as described by Logan et al. (Logan et al., 1999). These primary antibodies were detected with Oregon green-conjugated goat anti-rabbit secondary antibodies (Molecular Probes). Embryos were blocked in TBS-T (5 mM Tris-HCl, pH 7.5, 70 mM NaCl, 1.3 mM KCl, 0.5% Tween20) containing 40 mg/ml goat serum. For the staining with the Hpoe antibody (Yoshikawa, 1997), embryos were fixed as described by Coffman and McClay (Coffman and McClay, 1990) and the primary antibodies were detected with Texas Red-conjugated secondary antibodies (Molecular Probes).

Western analysis

Embryos were dissolved in sample buffer [final concentration: 290 mM Tris-HCl (pH 6.8), 8.3% SDS, 30% glycerol, 0.01% Bromphenol Blue, 4% 2-mercaptoethanol], and boiled for 5 minutes. Proteins were analysed on 8% acrylamide gels by SDS-PAGE and transblotted on to a PVDF membrane (Immobilon Transfer Membranes; Millipore). The membrane was reacted with affinity-purified polyclonal anti-HpTb antibodies, followed by horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:1000000; KPL), followed by detection with Super Signal West Dura Extended Duration Substrate (PIERCE) as an enzymatic substrate. The chemiluminescent signal was detected by X-ray film.

RT-PCR analysis

Total RNA was isolated from 50 control and 50 mRNA-injected embryos or 50 embryos derived from animal cap mesomeres using ISOGEN (Wako). The extracted RNAs were used to synthesize cDNA using RNA PCR kit (AMV) (Takara). An aliquot of the RT reaction was then used for PCR containing 0.2 μM concentrations of appropriate primers. All comparisons were performed in the linear

range of amplification. The products were resolved on 2% agarose gels and then transferred to a Nytran membrane (Schleicher and Schuell). To visualize the PCR products, hybridization with appropriate DIG-labelled RNA probes was followed by commercial Fab fragments of antibody to DIG conjugated to alkaline phosphatase (Roche). The chemiluminescent signal produced by enzymatic dephosphorylation of CSPD by alkaline phosphatase was detected by X-ray film.

Construction of mesomere-micromeres chimeras

Micromere and animal cap isolation, and cell transplantations were performed by hand using a glass needles as described by Kurokawa et al. (Kurokawa et al., 1999).

RESULTS

The sea urchin conserves a homologue of the mouse *T-brain* gene

Using oligonucleotide primers corresponding to a conserved sequence in the *T*-box gene family, we amplified target fragments from *H. pulcherrimus* gastrula cDNA by the PCR reaction. Sequencing the amplified 300 bp-long fragments revealed that the sea urchin contains a *T*-box gene different from *HpTa*, which we previously reported as a *brachyury* homologue (Harada et al., 1995). Thus, we designated this second *T*-box gene as *HpTb*. Screening hatched blastula cDNA library (*H. pulcherrimus*) with DIG-labelled RNA probe yielded a clone that consisted of 4975 nucleotides. As shown in Fig. 1, the cloned fragment contains a single open reading frame of 2817 nucleotides that encodes a polypeptide of 939 amino acids and a calculated molecular mass of 105 kDa.

We performed a molecular phylogenetic analysis using the 136 confidently aligned sites of the T-domain amino acid residues. The resultant phylogenetic tree indicates that *HpTb* is a *T*-box gene belongs to the subfamily of *T-brain* (Fig. 2A).

Fig. 2B shows a comparison of the amino acid sequences of the T domain of HpTb with proteins encoded by the human *hu-Tbr-1* (Bulfone et al., 1995), mouse *m-T-brain-1* (Bulfone et al., 1995), zebrafish *zf-tbr 1* (Yonei-Tamura et al., 1999), *X-Eomesodermin* (Ryan et al., 1996) and starfish *Ap-Tbr* (Shoguchi et al., 2000). Although the overall degree of amino acid identity is not very high, in the T domain shown in Fig. 2 the extent of amino acid identity was 61% (sea urchin/mouse), 60% (sea urchin/frog), 60% (sea urchin/zebrafish) and 72% (sea urchin/starfish). The relatively high degree of identity in the T domain between the sea urchin protein and the mouse *T-brain-1*, *X-Eomesodermin*, *ZF-tbr 1* and *Ap-Tbr* proteins demonstrates that this cDNA clone corresponds to a sea urchin homologue of the chordate *T-brain* gene.

Expression of *HpTb* during sea urchin embryogenesis is transient

Northern blotting analysis revealed that the *HpTb* transcripts are transiently present during embryogenesis of *H. pulcherrimus*. The probe produced from the entire cDNA for *HpTb* hybridized to a 6 kb RNA. Although the length of the cloned *HpTb* cDNA isolated from gastrula is ~5 kb, 6 kb RNA seems to be actual size mRNA of *HpTb*. The distinct hybridization signal for *HpTb* was first detected in blastulae and the level of the signal was almost constant until gastrula stage. Thereafter, the signal diminished rapidly (Fig. 3A).

The Northern blotting also detected a very weak band of about 4.5 kb in the egg and cleavage stages (Fig. 3A). We isolated the cDNA clones from cleavage stage embryos using the entire *HpTb* cDNA as a probe. Sequencing of these clones revealed that the mRNAs of the cleavage stage embryos are shorter, being truncated at the C-terminal region (Fig. 1).

HpTb-transcripts localize to differentiating PMCs

We studied which territories or cell types express *HpTb* by means of in situ hybridization of whole-mount specimens. At the hatched blastula stage, the distinct signal of *HpTb* was detected in the presumptive PMCs (Fig. 3B), which form a ring around vegetal pole (Fig. 3C). At the mesenchyme blastula stage, these *HpTb* positive cells migrate into the blastocoel to give rise to PMCs (Fig. 3D). The primary skeletogenic mesenchyme cells are derived from (large) micromeres. No hybridization signals were detected in embryonic cells other than PMCs. After the gastrula stage, the *HpTb* whole-mount hybridization signal is no longer detectable (data not shown).

HpTb localize to nucleus of PMCs after blastula stage

Western blot analysis, using affinity-purified anti-HpTb antibodies, revealed that HpTb protein is detected as a single band with an estimated molecular weight of 105 kDa. As the shorter, processed HpTb-mRNAs detected in the egg and cleavage stage embryos lack part of the coding region, we would expect the molecular mass of the proteins translated from cleavage stage HpTb-mRNAs to be smaller than the HpTb expressed after the blastula stage. However, no such smaller band was detected, suggesting that the processed HpTb-mRNA is not translated. The full-length HpTb protein is present in the egg and cleavage stage, decreases in abundance before hatching and then increases at the hatched blastula stage. Thereafter the level of protein remains almost constant until the pluteus stage (Fig. 4A). Taken together with the results of northern blots (Fig. 3A), the results indicate that the HpTb protein is accumulated maternally, destroyed before hatching, and then produced zygotically after the blastula stage.

Immunostaining of the embryos with anti-HpTb antibodies revealed that HpTb is present in the cytoplasm, but is absent from nuclei of all blastomeres in the cleavage stage. This suggests that the maternally stored HpTb does not function as a transcription factor (Fig. 4B). After the hatching blastula stage, the HpTb disappears from blastomeres except for PMCs, and HpTb is accumulated in the nuclei of (presumptive) PMCs (Fig. 4C).

Repression of *HpTb* causes significant delay of gastrulation

In order to gain the insight into the role of *HpTb* during development, we designed experiments to perturb the embryo by inhibiting the translation of HpTb by injecting fertilized eggs with *HpTb* morpholino antisense oligonucleotides. When the control *lacZ* morpholino (7×10^8 molecules/egg) or low amounts of *HpTb* morpholino (1×10^8 molecules/egg) were injected, most of the injected embryos developed almost normally to the pluteus stage (Fig. 5A,B). The embryos

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TGCAGCTTTATTTTTGACAGTTTCTTGCAAATTTGATGGTAGCTTGTCTCACGCCCAAAAGAGATGATCACATACAAATCGCACCAGGGGAATTTCGGAA  100
AAAGTGTCAAAATCGCAGTGAGACTTTCATCAGCGTTCGCGCCTTCTCGCTTCGGTGTTTATCCATGTAATTTGTGACTGAATTTTCGCACTCCGACTCT  200
AACTCCAATTTAAAGGGATTGAATTCTAACGCCTTCGCGCCTTTTGATTTTGGTGCGATTTTTCCACTTTGAGGAGACATGATGGGAGAAGAATTTCAAC  300
                                                                                 M  M  G  E  E  F  Q  H
ACACAACTGAACAGGGTAATGAACACGTTGCTGCAACAAATCCAACTAAGTTTCAAAAGACGGAACCAGAAGAAAGCGATGAAGTTTCTGAAGGTGAGAA  400
 T  T  E  Q  G  N  E  H  V  A  A  T  N  P  T  K  F  Q  K  T  E  P  E  E  S  D  E  V  S  E  G  E  N
TCTTGATGACGGGAGCTATGGATCTGAAGATACCAGCTGCGAGAAGTCAAAAGTCGAACAATTCCACACCAATGAATTAATTCCGAACGCTGATCAGAAC  500
 L  D  D  G  S  Y  G  S  E  D  T  S  C  E  K  S  K  V  E  Q  F  H  T  N  E  L  I  P  N  A  D  Q  N
GTCGGGGATCCAAATAACGACTACCCTTGCAACAAGTTTGATTTATGTAAGATCAAGTCCAATTTAGAGAATTCTCAAACAGGGAGGCAGTCTAATTTGT  600
V  G  D  P  N  N  D  Y  P  C  N  K  F  D  L  C  K  I  K  S  N  L  E  N  S  Q  T  G  R  Q  S  N  L  F
TTCAGAGGAATGGACATGGACATGGAAATGCAGAACAATCGTTTAGTTTTAATGATTTTAACGCACATCATGACTCGGATGGAAATTTGCAGAGGTATTG  700
  Q  R  N  G  H  G  H  G  N  A  E  Q  S  F  S  F  N  D  F  N  A  H  H  D  S  D  G  N  L  Q  R  Y  C
TCCGTTCCCGTCCCTGCAAAACCAACAGTCGAATTTACCGGAATACCTGTTGAATACTAATTGCACTCTCTCCAACTTCACAGGCATTCCAACGCCGCCG  800
 P  F  P  S  L  Q  N  Q  Q  S  N  L  P  E  Y  L  L  N  T  N  C  T  L  S  N  F  T  G  I  P  T  P  P
CCTGCCACTTCGCAAAGCCAACAGGTGCGTTTCGGGACGGCTCCTTATGCAGATGGGTTCCAGCACCTGTCCCAAACAAATCAAACCCACCGGCAAATAT  900
P  A  T  S  Q  S  Q  Q  V  R  F  G  T  A  P  Y  A  D  G  F  Q  H  L  S  Q  T  N  Q  T  H  R  Q  I  S
CGGAGATCGAAACCCCTTCAGTGAACGATGCACTGAACCATAATCTCCATGGTACTGGGTTTACACTGCACGGCAACTCGCAAACGACTTCTTCAGGTTG  1000
  E  I  E  T  P  S  V  N  D  A  L  N  H  N  L  H  G  T  G  F  T  L  H  G  N  S  Q  T  T  S  S  G  C
TAGCAACACGGATGAAAAACTTGCTACCAATCAAATTCAGCAACATCTGCAACAGCAGCAGCAATTAACGTTGCAGGATTTTGACATGAAGAAAGAACTC  1100
 S  N  T  D  E  K  L  A  T  N  Q  I  Q  Q  H  L  Q  Q  Q  Q  Q  L  T  L  Q  D  F  D  M  K  K  E  L
GAGCATGGTGATATCCGGGTACCGCCATTCTTTGTTGAAAACACACCACCGCTGCAACAGACGACAAACACCTTGCCACAGCAGCTCATTGGCGGCTCTT  1200
E  H  G  D  I  R  V  P  P  F  F  V  E  N  T  P  P  L  Q  Q  T  T  N  T  L  P  Q  Q  L  I  G  G  S  L
TGGGTCCGCCATCGTCCCATAGCTTCGGCCACCATCACAACAGAGCCTTGCACGAGCAGGAGGACGAGAACGCGAAAAGTCACCTCTCGAGTCACGTCGT  1300
  G  P  P  S  S  H  S  F  G  H  H  H  N  R  A  L  H  E  Q  E  D  E  N  A  K  S  H  L  S  S  H  V  V
CAATCAGCCGTACCCACCGCCAGCAGGACACCAGCATGTCGAGGTCGCCAATGGGCCGGGGAAGGCGTCGGTTTACCTCTGCAACCGAGACCTGTGGCGG  1400
 N  Q  P  Y  P  P  P  A  G  H  Q  H  V  E  V  A  N  G  P  G  K  A  S  V  Y  L  C  N  R  D  L  W  R
AAGTTTCATCAGCATAAGACAGAGATGATCATCACTAAACAAGGAAGGCGGATGTTTCCACAGCTCGTCTTCAAATTGACCGGTCTCAATCCAACCTCGC  1500
K  F  H  Q  H  K  T  E  M  I  I  T  K  Q  G  R  R  M  F  P  Q  L  V  F  K  L  T  G  L  N  P  T  S  Q
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  Y  N  V  F  V  D  M  V  L  C  D  P  N  Q  W  K  F  Q  C  G  K  W  I  P  C  G  Q  A  E  N  I  P  K
AGTAAGCAACATCTATCTTCATCCTGATTCACCTAGCAATGGACTGCATTGGATGCATCAAGACATCGTATTCAGTAAGCTCAAGCTGACCAACCATCGG  1700
 V  S  N  I  Y  L  H  P  D  S  P  S  N  G  L  H  W  M  H  Q  D  I  V  F  S  K  L  K  L  T  N  H  R
GCAAAAGACAATGGATTTGTGATCTTAAACTCGATGCACCAGTATCAGCCTCGAATTCACGTCCTAGAGTTGACCGAGAGCCGCTCTATCCAAACCCATA  1800
A  K  D  N  G  F  V  I  L  N  S  M  H  Q  Y  Q  P  R  I  H  V  L  E  L  T  E  S  R  S  I  Q  T  H  S
GCTTTCCCGAGACACAATTCTTCGCCGTGACGGCCTACCAAAATACTGACGTAACACAACTGAAAATCGACTACAATCCATTTGCAAAGGGTTTCAGAGA  1900
  F  P  E  T  Q  F  F  A  V  T  A  Y  Q  N  T  D  V  T  Q  L  K  I  D  Y  N  P  F  A  K  G  F  R  D
TAACTACGACAACTTGTCACCACGAGACATTAGTATCCTGTCGAATGCTCCCCGGTCTAAGAACGTAACGGTCAGCAGGAATTCCTGCCCTGCACCGGTA  2000
 N  Y  D  N  L  S  P  R  D  I  S  I  L  S  N  A  P  R  S  K  N  V  T  V  S  R  N  S  C  P  A  P  V
GTGAATGCAGCTAACATCGCCATGGGTCATTTTCCTGCCATGATCTCTCACCAGCCCGGTCACTATCAGCACCCGTACCACCACCACGCCCACCATGCCC  2100
V  N  A  A  N  I  A  M  G  H  F  P  A  M  I  S  H  Q  P  G  H  Y  Q  H  P  Y  H  H  H  A  H  H  A  H
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AGGCCAAGCTCCAGCGGGATGCAAAGCAGGTCCGACGGGCGGAAATTTCCGGCTTCCTAATCCAGACGAGACCGCTTTCAAGTTAGTGGGGAACGCCTCG  2300
 G  Q  A  P  A  G  C  K  A  G  P  T  G  G  N  F  R  L  P  N  P  D  E  T  A  F  K  L  V  G  N  A  S
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  I  V  Q  N  S  L  C  S  T  H  Q  G  Q  Q  M  L  Q  Q  Q  Q  H  D  K  K  Q  L  H  S  P  S  D  S  I
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ACTGCTCCCCGGATTCCAAGTCCAACTCGAGCGACAGCGGGGGTCAGGCTGCCAAACGCCAGAAGATGTCCGAGGAGCAGCTATCTGAAGGCGTCTCTCC  2800
  C  S  P  D  S  K  S  N  S  S  D  S  G  G  Q  A  A  K  R  Q  K  M  S  E  E  Q  L  S  E  G  V  S  P
AAAGCTGTCGGATCTTGGTGACCAACCCAACGGCGCCACCGGTTATGAGCTTACATCTACCATAGATCACGGCTATATCAACCAAGAGGGCGCCAAACAT  2900
 K  L  S  D  L  G  D  Q  P  N  G  A  T  G  Y  E  L  T  S  T  I  D  H  G  Y  I  N  Q  E  G  A  K  H
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D  Y  L  Q  S  I  H  A  Q  S  N  A  P  H  Q  V  F  Q  P  Y  G  N  M  Y  F  P  Q  S  F  P  S  F  P  S
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TATCACTGTTTGTTGTGTTGGATGTAAATTATTCATTGAACTGTTTCATTGTTTTTAAATAAATGCAAAGAGTTA                              4975
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Fig. 1. Nucleotide and deduced amino acid sequences of the cDNA clone for the sea urchin T-box gene, *HpTb*. The T-domain is underlined. The termination codon is shown with an asterisk, and the potential signal sequence for polyadenylation is double underlined. The position of 3′ end of cDNAs isolated from cleavage stage embryos is depicted with arrowheads. DDBJ Accession Number for *HpTb* is AB048760.

injected with 7×10^8 molecules of *HpTb* morpholino seemed to be morphologically normal until hatched blastula stage (data not shown). When control embryos, which were injected with *lacZ* morpholino, had reached the early gastrula stage (Fig. 5C), embryos injected with *HpTb* morpholino showed suppressed and delayed gastrulation; however, the ingression of PMCs into blastocoel occurred normally in such embryos (Fig. 5D). When the control embryos reached the prism stage (Fig. 5E), embryos injected with *HpTb* morpholino showed retarded gastrulation. Furthermore, the differentiation of oral-aboral ectoderm seemed to be repressed, and formation of spicules was also suppressed (Fig. 5F). As judged by cell morphology and their localization in the animal hemisphere, secondary mesenchyme cells (SMCs) were formed in 48 hour prism embryos injected with *HpTb* morpholino. At 72 hours after fertilization, when the control embryos had reached the pluteus stage, only a limited archenteron and a reduced number of pigment cells (approx. one-third compared with the control embryos) were observed in the embryo injected with *HpTb*-morpholino (Fig. 5H). Most of the embryos injected with 7×10^8 molecules (10 pg) of *HpTb* morpholino (n=256/267) showed this phenotype. We confirmed that the *HpTb* morpholino antisense oligonucleotides inhibited the translation of HpTb by immunostaining with anti-HpTb antibodies (Fig. 5I,J). These results suggest that the expression of *HpTb* in (developing) PMCs is required for the gastrulation, spicule formation and the normal development of the oral-aboral axis in sea urchin development. The formation of an archenteron was rescued in more than half of embryos injected with *HpTb* morpholino by co-injection of *HpTb* mRNA (n=72/128; Fig. 5K). However the inhibition of skeletogenesis was barely rescued by co-injection of *HpTb* mRNA. We cannot explain the reason for the inefficient rescue of skeletogenesis at this point.

Repression of *HpTb* causes suppression of *Ars* and *SM30*

We performed quantitative RT-PCR to determine the expression level of various cell type-specific genes in the embryos injected with *HpTb* morpholino. The RNA was isolated from embryos at 28 hours after fertilization, when the control embryos had reached late gastrula stage. As a control,

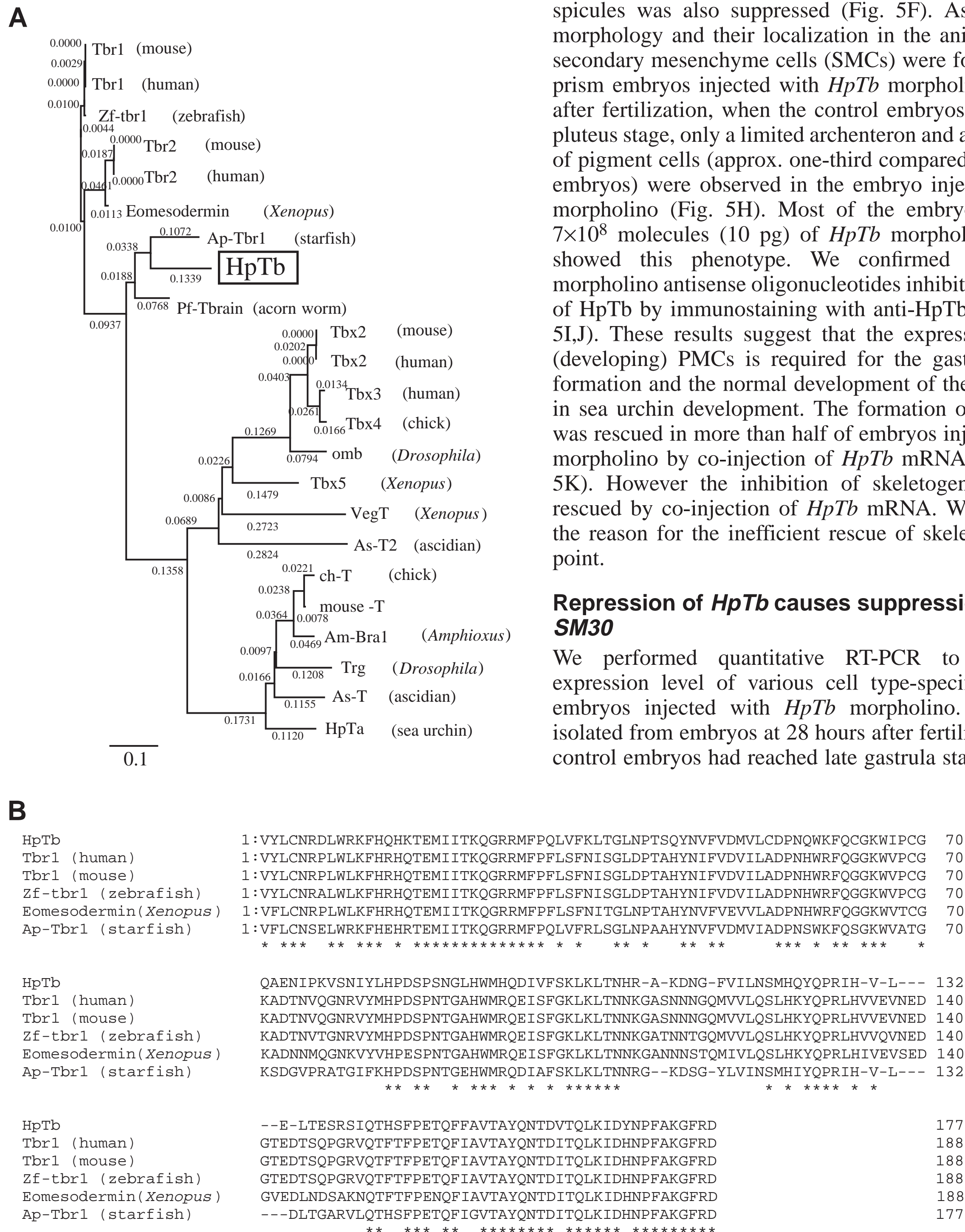

Fig. 2. Relationships of sea urchin T-domain proteins. (A) A phylogenetic tree of T domains. A molecular phylogenetic tree was constructed using the NJ method by a computer program (Genetyx, Software Development). The branch length is proportional to the number of amino acid substitutions. The number at each branch indicates the values of expected substitution of amino acid residues. (B) Alignment of the amino acid sequences of the T domain of T-brain subfamily members. Asterisks (*) indicate that all of the six T-brain proteins have an identical amino acid.

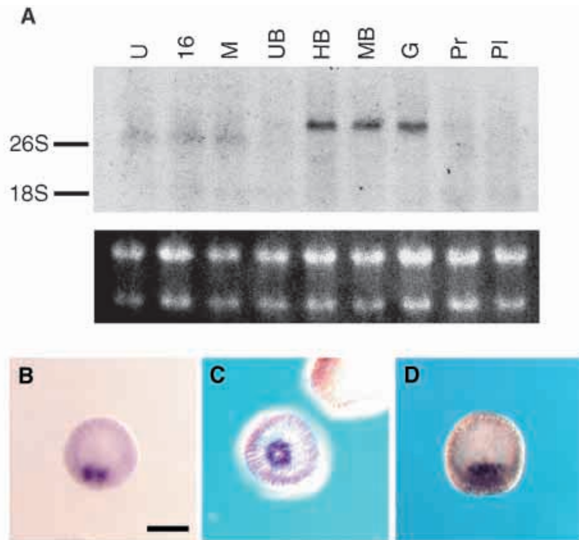

Fig. 3. *HpTb* is activated zygotically and is expressed transiently during the blastula and gastrula stages. (A) Developmental northern blot. Two micrograms of total RNA extracted from sea urchin embryos at various developmental stages were run in parallel. The lower panel shows the rRNA bands in the ethidium bromide-stained gel. U, unfertilized egg; 16, 16-cell stage; M, morula; UB, unhatched blastula; HB, hatched blastula; MB, mesenchyme blastula; G, gastrula; Pr, prism; Pl, pluteus. (B-D) Whole-mount in situ hybridization showing spatial expression of *HpTb* transcript during embryogenesis. (B,C) Hatched blastulae, (B) lateral view and (C) vegetal view. (D) Early mesenchyme blastula, lateral view. PMCs migrating into the blastocoel show a positive signal. Scale bar: 50 μm.

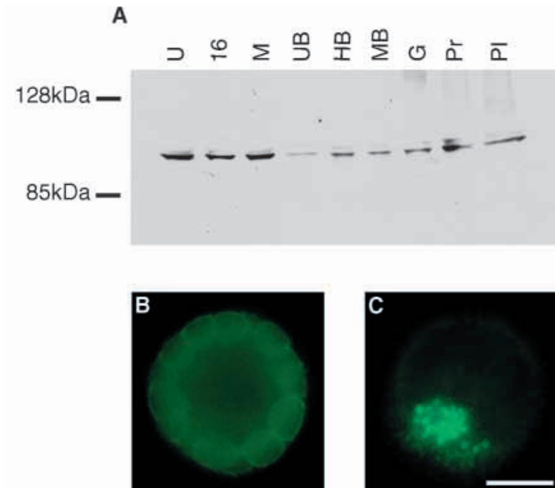

Fig. 4. HpTb protein is accumulated into PMC nucleus after blastula stage. (A) Developmental western blot. Thirty micrograms of protein prepared from embryos at various developmental stages were run in parallel. Detection procedure of HpTb protein is described in text. U, unfertilized egg; 16, 16-cell stage; M, morula; UB, unhatched blastula; HB, hatched blastula; MB, mesenchyme blastula; G, gastrula; Pr, prism; Pl, pluteus. (B,C) Immunostaining of embryos with antibodies to HpTb. (B) Morula; (C) mesenchyme blastula. Scale bar: 50 μm.

the level of ubiquitin mRNA, which is almost spatially ubiquitous (Nemer et al., 1991), was unaffected by injection of the *HpTb* morpholino.

We previously showed that *HpEts*, an ets-related transcription factor, is expressed exclusively in micromere descendants after blastula stage, and that *HpEts* is involved in the differentiation of PMCs (Kurokawa et al., 1999). Recently, Sweet et al. (Sweet et al., 2002) reported that a sea urchin *Delta* homolog (*LvDelta*) is also expressed in micromere descendants at blastula stage, and that *LvDelta* is responsible for the SMC-inducing activity of micromere descendants. In order to determine the functional relationship of *HpTb* to *HpEts* and *HpDelta*, we examined the expression of *HpEts* and *HpDelta* in embryos injected with *HpTb* morpholino. As shown in Fig. 6, *HpEts* and *HpDelta* were unaffected in the injected embryos. This was supported by the observation that PMCs, which ingressed into the blastocoel of embryos injected with *HpTb* morpholino, were immunologically positive using the anti-HpEts antibodies (Fig. 7B). Furthermore, some pigment cells, which are derived from SMCs, formed in morpholino injected embryos. In addition, the expression of PMC-specific *HpSM50* was not affected by the injection with *HpTb* morpholino (Fig. 6). These results suggest that *HpTb* is not involved in the specification of PMCs. By contrast, another PMC-specific gene *HpSM30* (Kitajima et al., 1996) was suppressed in the embryos injected with *HpTb* morpholino (Fig. 6). It has been reported that expression of *SM30* requires signal(s) from non-PMCs (Urry et al., 2000). It is possible that HpTb is indirectly involved in the production of signal(s) responsible for the expression of *HpSM30*.

Expression of the aboral ectoderm-specific gene *HpArs* (Akasaka et al., 1990) was also suppressed in the embryos injected with *HpTb* morpholino, whereas Hpoe, which is an oral ectoderm-specific epitope (Yoshikawa, 1997), was expressed over almost all the surface of epithelial cells of the injected embryos (Fig. 7D). Wikramanayake et al. have shown that the activation of aboral ectoderm-specific *Spec1* in *L. pictus* requires signals from vegetal hemisphere (Wikramanayake et al., 1995). Recent studies also suggest that vegetal signals are involved in the establishment of oral-aboral axis (Wikramanayake and Klein, 1997; Li et al., 1999; Angerer and Angerer, 2000). In order to confirm that the activation of aboral ectoderm-specific *Ars* also requires interaction with vegetal blastomeres in *H. pulcherrimus* embryos, we performed quantitative RT-PCR to determine the expression level of *Ars* in the embryos derived from animal cap mesomeres. The RNA was isolated from control embryos and from embryos derived from animal cap mesomeres at 28 hours after fertilization when the control embryo had reached the late gastrula stage. The expression level of the *Ars* was significantly lower in the embryo derived solely from animal cap mesomeres, suggesting that signals from vegetal hemisphere are required for the activation of *Ars* (Fig. 8). These results raise a possibility that HpTb is involved in the production of vegetal signal(s) involved in aboral ectoderm differentiation.

The levels of *HpTa* (Harada et al., 1995) and *HpEndo16* (Akasaka et al., 1997), both of which are expressed in the vegetal plate at blastula stage, were not affected by the injection of *HpTb* morpholino at the developmental stage we examined, suggesting that HpTb is not involved in the initial specification of endoderm. It is important to note that the level

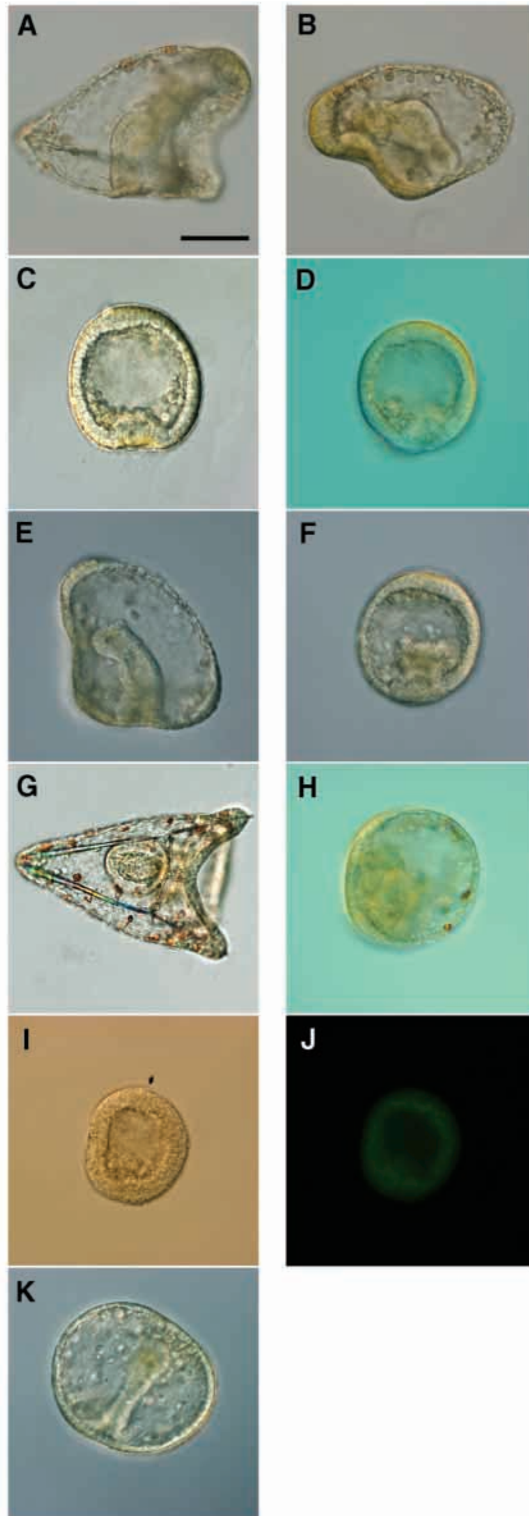

Fig. 5. Effect of injection of *HpTb* morpholino antisense oligonucleotides on the embryogenesis. (A,C,E,G) Control embryos injected with *lacZ* morpholino (7×10^8 molecules/egg). (B) Embryo injected with low amounts of *HpTb* morpholino (1×10^8 molecules/egg). (D,F,H-J) Embryos injected with *HpTb* morpholino (7×10^8 molecules/egg). The repression of translation of HpTb was confirmed using indirect immunofluorescence with anti-HpTb antibodies on mesenchyme blastula, (I) bright-field view, (J) epifluorescence view. (K) Embryo co-injected with 1.5 pg of *HpTb*-mRNA and *HpTb* morpholino (7×10^8 molecules/egg). Embryos at 28 hours (C,D,I,J), 40 hours (E,F), 48 hours (A,B,K) and 72 hours (G,H). Scale bar: 50 μm.

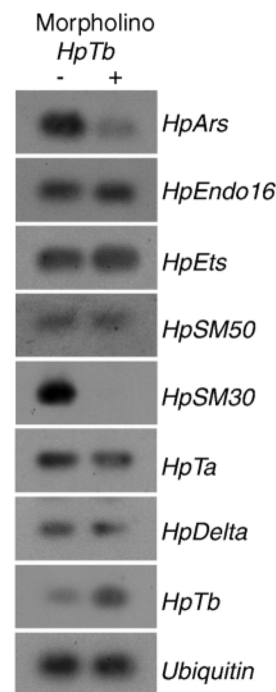

Fig. 6. RT-PCR analysis of various tissue specific genes in control and embryos injected with *HpTb* morpholino. Total RNA was isolated from 50 control embryos and 50 embryos injected with *HpTb* morpholino antisense oligonucleotides at 28 hours after fertilization. Southern blotting of RT-PCR products. (–) and (+) indicate uninjected and injected embryos. The genes analysed are discussed in the text.

of expression of *HpTb* was enhanced by the repression of translation of HpTb (Fig. 6).

Repression of HpTb-translation diminishes the ability to signal to neighbours on PMCs

Micromere-progeny induction signals have been shown to play an important role in the specification of SMCs (McClay et al., 2000) and initiation of gastrulation (Minokawa and Amemiya, 1999; Ishizuka et al., 2001). As repression of *HpTb* in (presumptive) PMCs leads a significant delay of gastrulation, we predicted that *HpTb* might be involved in regulating (presumptive) PMC signaling. To test this hypothesis, we prevented the translation of HpTb in micromere descendant cells by injecting *HpTb* morpholino antisense oligonucleotides. We combined animal cap mesomeres from a normal embryo with a micromere quartet isolated from a 16-cell stage embryo that had developed from a zygote injected with 7×10^8 molecules of *HpTb* morpholino (Fig. 9A,B). In order to follow HpTb deficient micromeres, donor embryos were labelled by co-injecting the morpholino with 10 pg of rhodamine-dextran. In all experiments, over 100 injected embryos were also cultured in parallel for 48 hours to confirm that the archenteron formation was suppressed in the donor embryos.

When *HpTb* morpholino-injected donor micromeres were combined with the animal cap mesomeres of uninjected embryo, almost all the transplanted micromere descendant cells ingressed into the blastocoel, but the injected donor micromeres only induced a very limited archenteron, and the micromeres did not form spicules (n=5/5; Fig. 9B). SMCs were

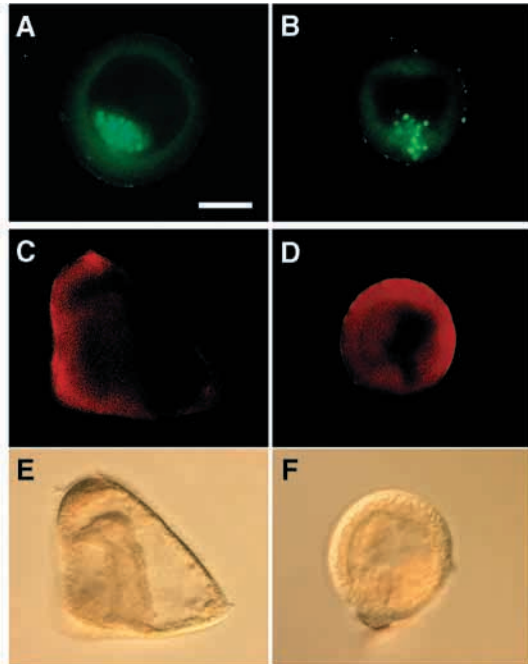

Fig. 7. Localization of PMC specific HpEts and oral ectoderm specific Hpoe with antibodies. (A,C,E) Control embryo and (B,D,F) embryo injected with *HpTb* morpholino. (A,B) Embryos stained with antibodies to HpEts at 23 hours after fertilization; (C,D) embryos stained with antibodies to Hpoe at 40 hours after fertilization; (E,F) phase image of C,D. Scale bar: 50 μm.

either not formed at all, or very small number of SMCs were formed, in the chimaeric embryos. By contrast, when uninjected micromeres were combined with animal cap mesomeres of uninjected embryo, the micromeres ingressed and formed spicules (*n*=15/15). In addition, the control donor micromeres were able to induce an archenteron, oral-aboral ectoderm and SMCs (*n*=15/15; Fig. 9C). These data suggest that one of the functions of HpTb is to provide the micromere descendant cells with the ability to produce a signal that induces neighbouring cells to develop archenteron and SMCs. When the animal cap mesomeres derived from zygotes injected with the morpholino antisense oligonucleotides were combined with micromere quartet derived from normal embryos, the mesomeres developed archenteron, SMCs and both oral and aboral ectoderms (*n*=14/14; 9D,E). The micromere progeny developed spicules in the chimaeric embryos. These data support the hypothesis that *HpTb* morpholino antisense oligonucleotides do not affect the

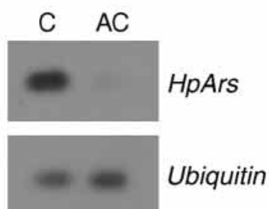

Fig. 8. RT-PCR analysis of *Ars* in embryos derived from animal cap mesomeres and control embryos. The RNA was isolated from 50 embryos derived from animal cap mesomeres at 28 hours after fertilization when the control embryo had reached the late gastrula stage. Southern blotting of RT-PCR products. C, control embryos; AC, embryos derived from animal cap mesomeres.

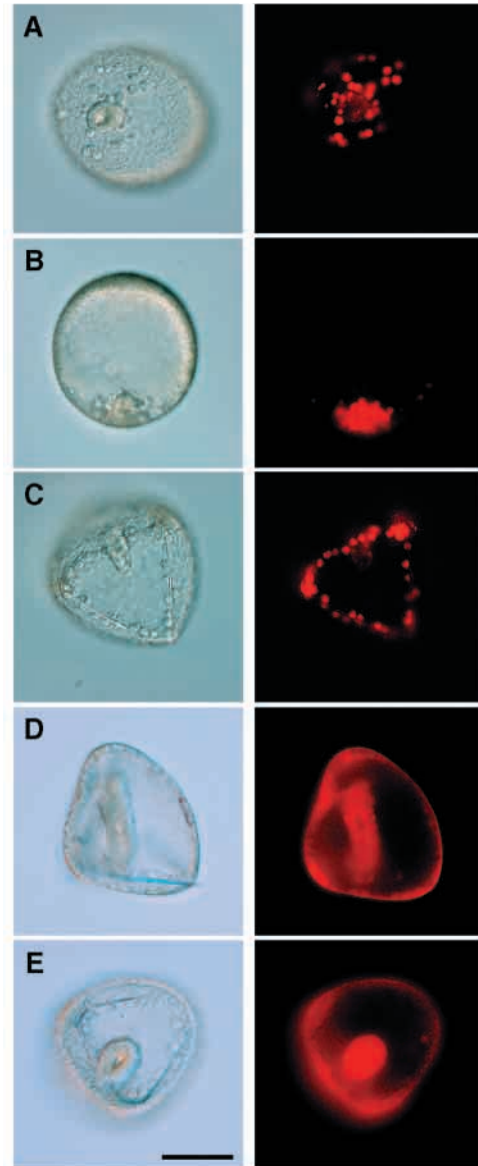

Fig. 9. Loss of organizing activity of micromeres by injecting with *HpTb* morpholino. (A,B) Chimera composed of animal cap mesomeres from normal embryo with the micromere quartet isolated from a 16-cell stage embryo developed from zygotes injected with 7×10^8 molecules of *HpTb* morpholino (A, vegetal view; B, lateral view). (C) Control embryo. (D,E) The micromeres were labelled with rhodamine-dextran. Chimera composed of micromere quartet from normal embryo with the animal cap mesomeres isolated from a 16-cell stage embryo developed from zygotes injected with 7×10^8 molecules of *HpTb* morpholino (D, lateral view; E, vegetal view). The animal cap mesomeres were labelled with rhodamine-dextran. Left, bright-field views; right, epifluorescence views. Scale bar: 50 μm.

responsiveness of mesomeres to the signals emanating from micromere progeny.

HpTb expression is regulated by *HpEts* and Wnt signalling cascade

Recent studies provide convincing evidence that β-catenin, a molecule of Wnt signaling cascade, plays an essential role in

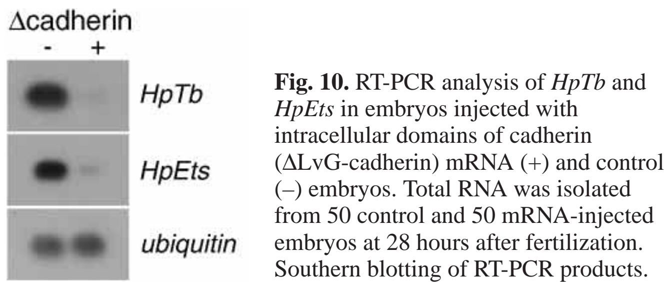

Fig. 10. RT-PCR analysis of *HpTb* and *HpEts* in embryos injected with intracellular domains of cadherin (ΔLvG-cadherin) mRNA (+) and control (–) embryos. Total RNA was isolated from 50 control and 50 mRNA-injected embryos at 28 hours after fertilization. Southern blotting of RT-PCR products.

specification of micromere-derived PMCs (Wikramanayake et al., 1998; Logan et al., 1999). Because *HpTb* is expressed exclusively in the (presumptive) PMCs, it is probable that the *HpTb* expression is regulated by nuclear localization of β-catenin. In order to examine this issue, we performed overexpression of intracellular domains of cadherin (ΔLvG-cadherin) to deplete β-catenin from the nuclei of blastomeres of early embryos. We injected 2 pg of Δ*LvG-cadherin* mRNA; this has been shown to abolish vegetal development of *Lytechinus* embryo (Logan et al., 1999). As shown in Fig. 10, this injection of Δ*LvG-cadherin* mRNA resulted in the suppression of *HpTb*; the *HpTb* band is barely detectable in experimental embryos. This is consistent with the idea that *HpTb* is in a downstream component of a Wnt signalling cascade that functions in the micromere primary mesenchyme lineage of the sea urchin embryo. We examined the expression of *HpEts*, which is also expressed exclusively in the (presumptive) PMCs after blastula stage, in the embryo injected with ΔLvG-cadherin mRNA. The expression of *HpEts* was also repressed in these injected embryos (Fig. 10). Thus, we might assume that *HpTb* and *HpEts* are in the same cascade of gene regulation. We examined the functional relationship of *HpTb* to *HpEts*. PMC-specific expression of *HpEts* was not affected by the injection with *HpTb* morpholino (Fig. 6, Fig. 7B). Conversely, the overexpression of the dominant negative, ΔHpEts, suppressed the expression of *HpTb* (Fig. 11A). These results suggest that both *HpEts* and *HpTb* are downstream components of the Wnt signalling cascade, and that *HpTb* is regulated by *HpEts*. The expression of dominant negative ΔHpEts also resulted in a significant delay and repression of gastrulation, as well as the suppression of differentiation of PMCs (Fig. 11C).

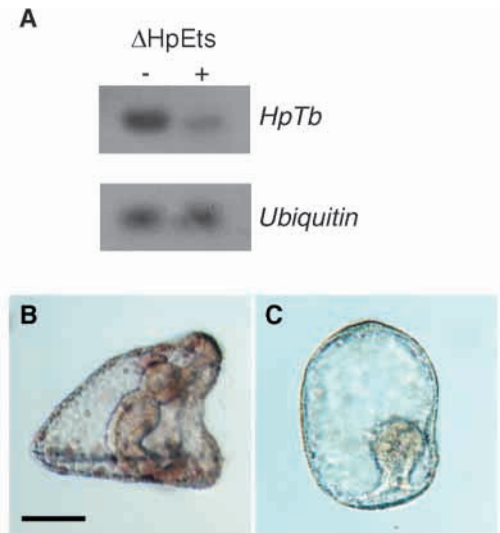

Fig. 11. Effect of the ectopic expression of ΔHpEts on the expression of *HpTb* and gastrulation. (A) RT-PCR analysis of *HpTb* in embryos injected with ΔHpEts (+) mRNA and control (–) embryos. Total RNA was isolated from 50 control and 50 mRNA-injected embryos at 28 hours after fertilization. Southern blotting of RT-PCR products. (B) Control embryo and (C) embryo injected with ΔHpEts-mRNA at 48 hours after fertilization. Scale bar: 50 μm.

DISCUSSION

The sea urchin conserves the *T-brain* gene

As shown in the present study, the *T-brain* gene is conserved in the sea urchin embryo and its expression is restricted to PMCs. The mouse T-Brain-1 gene (*Tbr1* – Mouse Genome Informatics) was initially isolated from a subtracted cDNA library enriched for genes transcribed at higher levels in the stage E14.5 mouse telencephalon than in the adult telencephalon (Bulfone et al., 1995). Examination by in situ hybridization of mouse T-Brain-1 gene expression demonstrated that the transcript is first detected around E10.5 in the mantle zone of the telencephalon, and defines molecularly distinct domains within the cerebral cortex (Bulfone et al., 1995). Interestingly, molecular phylogenetic analyses based on comparison of the T-domain amino acid sequences suggest that *Eomesodermin* should be included in the T-Brain subfamily (Fig. 2) (Papaioannou and Silver, 1998). *Eomesodermin* was originally isolated from *Xenopus laevis* embryos as a novel T-box gene (Ryan et al., 1996). The gene is expressed in mesodermal cells in a ventral-to-dorsal gradient of increasing concentration during gastrulation, and it is critical for mesoderm differentiation (Ryan et al., 1996), and later it was revealed to be expressed in the forebrain of tadpoles (Ryan et al., 1998). Recently, a mouse homologue (*Eomes*; also known as *Tbr2*) of eomesodermin was shown to be expressed in the early primitive streak, nascent mesoderm and the anterior visceral endoderm. This early expression disappears at later stages, and a second domain of *Eomes* expression is observed in the telencephalon around E10.5 (Ciruna and Rossant, 1999).

Homologs of the *Tbr1* gene have been identified from hemichordates (Tagawa et al., 2000), starfish (Shoguchi et al., 2000) and sea cucumbers (Maruyama, 2000). In these deuterostome embryos, *T-brain* is first expressed in the region which eventually forms endoderm and mesoderm. The hemichordate *T-brain* is expressed later in the apical organ or light sensory organ (Tagawa et al., 2000). Therefore, it is likely that the *T-brain* gene has two distinct expression domains: in the mesoendoderm of early embryos and in the nervous system of later embryos. The sea urchin *HpTb* expression in the micromere may correspond to the first expression domain of this family, and analogous to Eomesodermin, *HpTb* is likely to be critical for endoderm and mesoderm differentiation.

Developmental roles of the *HpTb* in sea urchin embryogenesis

Inhibition of translation of specific gene products with morpholine-substituted antisense oligonucleotides is a useful way to gain insight into the function of the gene (Howard et al.,

2001). *HpTb* is expressed specifically in (presumptive) PMCs during blastula and mesenchyme blastula stage. The expression pattern of *HpTb*, and suppression of archenteron formation caused by inhibition of the HpTb translation in (presumptive) PMCs are both consistent with what is known of the importance of presumptive PMCs in development (Minokawa and Amemiya, 1999; Ishizuka et al., 2001). Not only do micromeres provide cells for the skeleton, but it is known micromere descendants are an important source of developmental signals that affect other tissues. Removal of micromeres during the period from forth and fifth cleavage impairs expression of the endoderm specific *Endo16* and results in significant delay of archenteron formation (Ransick and Davidson, 1995). Recently, it has been shown that signal(s) emanating from micromere-descendants at late blastula stages are important for gastrulation itself (Minokawa and Amemiya, 1999; Ishizuka et al., 2001). Interference with (presumptive) PMC function by inhibiting translation of HpTb with *HpTb* morpholino oligonucleotides led to significant delay of gastrulation, as we report here. This is consistent with the known important role of micromere-descendants in these processes.

The repression of *HpTb* did not cause the inhibition of *HpEndo16* expression. This is also consistent with the previous report that the expression of *Endo16* is induced by micromere-descendant cells during forth to sixth cleavage stages (Ransick and Davidson, 1995). It seems likely that at least three distinct signals are provided by micromere descendant cells. The first signal(s) is produced during forth to sixth cleavage stages, as Ransick and Davidson reported (Ransick and Davidson, 1995); the second signal is Delta which is responsible for the SMC specification (McClay et al., 2000; Sweet et al., 2002) and the third signal(s) which is produced at blastula stage when the *HpTb* is expressed (Minokawa and Amemiya, 1999; Ishizuka et al., 2001). The present data favour the idea that *HpTb* is involved in gastrulation itself, but not in the initial specification of the vegetal plate.

The development of embryos from aggregates between mesomeres and micromeres (Hörstadius, 1973; Amemiya, 1996) demonstrates the organizing activity of micromeres. Normally, the mesomeres isolated from the 16-cell stage embryo form thin an epithelial ball (Hörstadius, 1973; Henry et al., 1989). Chimeras composed of animal cap mesomeres and micromere quartet from normal embryo developed almost normal embryos containing an archenteron, PMCs and SMCs. Conversely, *HpTb* morpholino-injected donor micromere quartet combined with the animal cap mesomeres of uninjected embryo induced only partially invaginated archenteron, and no or few SMCs (although the donor micromere descendant cells ingressed into blastocoel) (Fig. 9A,B). These results strongly support the idea that *HpTb* is involved in the regulation of signal(s) from (presumptive) PMCs. Although the chimeras composed of animal cap mesomeres and micromere quartet derived from zygotes injected with *HpTb* morpholino formed no or few SMCs, the embryos injected with *HpTb* morpholino formed SMCs (Fig. 5F,H) and expressed *HpDelta* (Fig. 6), suggesting that *HpTb* is not substantially involved in the specification of SMCs in normal development. Injection of *HpTb* morpholino resulted in the decreased number (approx. one third) of pigment cells. This raises the possibility that *HpTb* is involved in the differentiation of pigment cells indirectly.

Croce et al. have reported that the *T-brain* homolog referred to as *ske-T* is expressed in *P. lividus* embryos (Croce et al., 2001). They showed that transcripts hybridized with probes that detect *ske-T* exist ubiquitously in egg and the early cleavage stage embryos. They also showed that the transcripts appear after blastula stage, as we have shown in the present study. As Croce et al. pointed out, the early transcripts are smaller than the *ske-T* cDNA fragment they isolated (Croce et al., 2001). In our present work, we barely detected the small transcript in the eggs and cleavage stage embryos of *H. pulcherrimus*. We have also shown that the early transcripts are not translated (Fig. 4A) and that the maternally stored HpTb protein is not present in the nucleus, suggesting that the HpTb does not function as a transcription factor during cleavage stage (Fig. 4B). Furthermore, injection of *HpTb* morpholino antisense oligo into embryos of *H. pulcherrimus* did not affect early development.

When the animal cap mesomeres derived from zygotes injected with the morpholino antisense oligo were combined with a micromere quartet isolated from normal embryos, the mesomeres developed an archenteron, oral and aboral ectoderm and SMCs. Hence, even if low level of processed *T-brain* transcripts exist in the embryonic cells other than progeny of micromeres, they are not involved in the archenteron inducing activity, they are probably not responsible for the differentiation of oral-aboral ectoderm in *H. pulcherrimus*. It is also possible, of course, that differences exist between *H. pulcherrimus* and *P. lividus*.

Wikramanayake et al. (Wikramanayake et al., 1995) have shown that the aboral ectoderm specific genes were not expressed in animal hemisphere explants from *L. pictus*, suggesting that the ectoderm differentiation in *L. pictus* embryos requires interaction with vegetal blastomeres. Using *H. pulcherrimus* embryos, we also demonstrated that the embryos derived from the animal cap mesomeres did not express aboral ectoderm specific *Ars* (Fig. 8). The repression of translation of HpTb in (presumptive) PMCs resulted in the disturbances of patterning of oral and aboral ectoderm (overexpression of Hpoe epitope, reduction of *Ars* expression). It is possible that *HpTb* is involved in the production of the vegetal signals responsible (at least in part) for patterning oral and aboral ectoderm, although we do not know if the *HpTb* functions in this process directly or not.

HpTb morpholino oligonucleotides did not suppress the expression of *HpSM50*, *HpDelta* and *HpEts*, suggesting that *HpTb* is not involved in the specification of PMCs. In the previous paper, we have demonstrated that ectopic expression of *HpEts* altered the fate of many cells, transforming them into migrating PMCs. However, the transformed PMCs did not form spicules without addition of serum (Kurokawa et al., 1999). The expression of *HpEts* is necessary for the spicule formation, but it is not sufficient for the spicule formation of PMCs. It has been reported that another PMC-specific protein, *SM30*, requires signals produced by non-PMC cells (Urry et al., 2000). The repression of *HpSM30* after injection with *HpTb* morpholino (Fig. 6) supports the idea that the differentiation of vegetal regions, which are induced by a signal(s) emanated from (presumptive) PMCs, is required for the production of signal(s) responsible for the spicule formation.

Cascade of the *HpTb* expression

We have shown that both *HpEts* and *HpTb* are downstream

components of the Wnt signalling cascade. The injection of *HpTb* morpholino antisense oligonucleotides did not affect the expression of *HpEts*. Conversely, the overexpression of dominant negative ΔHpEts repressed the expression of *HpTb*, the gastrulation and the differentiation of oral-aboral ectoderm, suggesting that *HpEts* regulates *HpTb*. The injection of the *HpTb* morpholino also did not affect the expression of *HpDelta*, suggesting that *HpDelta* is not a downstream component of *HpTb*.

The repression of translation of HpTb caused enhancement of *HpTb* expression. There is experimental evidence that mid-cleavage stage blastomeres have an extensive capacity to change their states of specification in response to cell interactions (reviewed by Davidson, 1989). It has been thought that micromere descendant cells repress the capacity of neighbouring macromeres to change their cell fate into micromere progeny. It is possible that the expression of *HpTb* in (presumptive) PMCs downregulates the expression of *HpTb* in neighbouring cells.

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REFERENCES

Akasaka, K., Ueda, T., Higashinakagawa, T., Yamada, K. and Shimada, H. (1990). Spatial pattern of arylsulfatase mRNA expression in sea urchin embryo. *Dev. Growth Differ.* **32**, 9-1.

Akasaka, K., Frudakis, T. N., Killian, C. E., George, N. C., Yamasu, K., Khaner, O. and Wilt, F. H. (1994). Genomic organization of a gene encoding the spicule matrix protein SM30 in the sea urchin *Strongylocentrotus purpuratus*. *J. Biol. Chem.* **269**, 20592-20598.

Akasaka, K., Uemoto, H., Wilt, F., Mitsunaga-Nakatsubo, K. and Shimada, H. (1997). Oral-aboral ectoderm differentiation of the sea urchin embryos is disrupted in response to calcium ionophore. *Dev. Growth Differ.* **39**, 373-379.

Amemiya, S. (1996). Complete regulation of development throughout metamorphosis of sea urchin embryos devoid of macromeres. *Dev. Growth Differ.* **38**, 465-476.

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

Benson, S., Sucov, H., Stephens, L., Davidson, E. and Wilt, F. (1987). A lineage-specific gene encoding a major matrix protein of the sea urchin embryo spicule. I. Authentication of the cloned gene and its developmental expression. *Dev. Biol.* **120**, 499-506.

Bulfone, A., Smiga, S. M., Shimamura, K., Peterson, A., Puelles, L. and Rubenstein, J. L. R. (1995). *T-Brain-1*: A homolog of *Brachyury* whose expression defines molecularly distinct domains within the cerebral cortex. *Neuron* **15**, 63-78.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Ciruna, B. G. and Rossant, J. (1999). Expression of the T-box gene *Eomesodermin* during early mouse development. *Mech. Dev.* **81**, 199-203.

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.

Cox, K. H., DeLeon, D. V., Angerer, L. M. and Angerer, R. C. (1984). Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. *Dev. Biol.* **101**, 485-502.

Croce, J., Lhomond, G., Lozano, J. and Gache, C. (2001) ske-T, a T-box gene expressed in the skeletogenic mesenchyme lineage of the sea urchin embryo. *Mech. Dev.* **107**, 159-162.

Davidson, E. H. (1989). Lineage-specific gene expression and the regulative capacities of the sea urchin embryo: a proposed mechanism. *Development* **105**, 421-445.

Davidson, E. H., Cameron, R. A. and Ransick, A. (1998). Specification of cell fate in the sea urchin embryo: summary and some proposed mechanisms. *Development* **125**, 3269-3290.

Emily-Fenouil, F., Ghiglione, C., Lhomond, G., Lepage, T. and Gache, C. (1998). GSK3β/shaggy mediates patterning along the animal-vegetal axis of the sea urchin embryo. *Development* **125**, 2489-2498.

Frudakis, T. N. and Wilt, F. (1995). Two cis elements collaborate to spatially repress transcription from a sea urchin promoter. *Dev. Biol.* **172**, 230-241.

Gan, L., Wessel, G. M. and Klein, W. H. (1990). Regulatory elements from the related *Spec* genes of *Strongylocentrotus purpuratus* yield different spatial patterns with a lacZ reporter gene. *Dev. Biol.* **142**, 346-359.

George, N. C., Killian, C. E. and Wilt, F. H. (1991). Characterization and expression of a gene encoding a 30.6-kDa *Strongylocentrotus purpuratus* spicule matrix protein. *Dev. Biol.* **147**, 334-342.

Harada, Y., Yasuo, H. and Satoh, N. (1995). A sea urchin homologue of the chordate *Brachyury* (*T*) gene is expressed in the secondary mesenchyme founder cells. *Development* **121**, 2747-2754.

Harlow, E. and Lane, D. (1988). *Antibodies*: *A Laboratory Manual.* Cold Spring Harbor Laboratory Press.

Henry, J. J., Amemiya, S., Wray, G. A. and Raff, R. A. (1989). Early inductive interactions are involved in restricting cell fates of mesomeres in sea urchin embryos. *Dev. Biol.* **136**, 140-153.

Herrmann, B. G. and Kispert, A. (1994). The T genes in embryogenesis. *Trends. Genet.* **10**, 280-286.

Herrmann, B. G., Labeit, S., Poustka, A., King, T. R. and Lehrach, H. (1990). Cloning of the T gene required in mesoderm formation in the mouse. *Nature* **343**, 617-622.

Hörstadius, S. (1973). *Experimental Embryology of Echinoderms*. Clarendon Press, Oxford.

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 is sea urchin embryos. *Development* **128**, 365-375.

Ishizuka, Y., Minokawa, T. and Amemiya, S. (2001). Micromere-descendants at the blastula stage are involved in normal archenteron formation in sea urchin embryos. *Dev. Genes Evol.* **211**, 83-88.

Kitajima, T., Tomita, M., Killian, C. E., Akasaka, K. and Wilt, F. H. (1996). Expression of spicule matrix protein gene *SM30* in embryonic and adult mineralized tissues of sea urchin *Hemicentrotus pulcherrimus*. *Dev. Growth Differ.* **38**, 687-695.

Kurokawa, D., Kitajima, T., Mitsunaga-Nakatsubo, K., Amemiya, S., Shimada, H. and Akasaka, K. (1999). *HpEts*, an ets-related transcription factor implicated in primary mesenchyme cell differentiation in the sea urchin embryo. *Mech. Dev.* **80**, 41-52.

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 β-catenin signaling. *Development* **212**, 425-439.

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

Makabe, K. W., Carmen, K. V., Britten, R. J. and Davidson, E. H. (1995). Cis-regulatory control of the *SM50* gene. an early marker of skeletogenic lineage specification in the sea urchin embryo. *Development* **121**, 1957-1970.

Maruyama, Y. K. (2000). A sea cucumber homolog of the mouse *T-brain-1* is expressed in the invaginated cells of the early gastrula in *Holothuria leucospilota*. *Zool. Sci.* **17**, 383-387.

McClay, D. R., Peterson, R. E., Range, R. C., Winter-Vann, A. M. and Ferkowicz, M. J. (2000). A micromere induction signal is activated by β-catenin and acts through notch to initiate specification of secondary

mesenchyme cells in the sea urchin embryo. *Development* **127**, 5113-5122.

Minokawa, T. and Amemiya, S. (1999). Timing of the potential of micromere- descendants in echinoid embryos to induce endoderm differentiation of mesomere-descendants. *Dev. Growth Differ*. **41**, 535-547.

Nemer, M., Rondinelli, E., Infante, D. and Infante, A. A. (1991). Polyubiquitin RNA characteristics and conditional induction in sea urchin embryos. *Dev. Biol*. **145**, 255-265.

Okazaki, K. (1975). Spicule formation by isolated micromeres of the sea urchin embryo. *Am. Zool*. **15**, 567-581.

Papaioannou, V. E. and Silver, L. M. (1998). The T-box gene family. *BioEssays*. **20**, 9-19.

Ransick, A. and Davidson, E. H. (1993). A complete second gut induced by transplanted micromeres in the sea urchin embryo. *Science* **259**, 1134-1138.

Ransick, A. and Davidson, E. H. (1995). Micromeres are required for normal vegetal plate specification in sea urchin embryos. *Development* **121**, 3215-3222.

Ryan, K., Garrett, N., Mitchell, A. and Gurdon, J. B. (1996). *Eomesodermin*, a key early gene in *Xenopus* mesoderm differentiation. *Cell* **87**, 989-1000.

Ryan, K., Butler, K., Bellefroid, E. and Gurdon, J. B. (1998). *Xenopus eomesodermin* is expressed in neural differentiation. *Mech. Dev*. **75**, 155-158.

Sanger, F., Nicklen, S. and Coulson, A. R. (1992). DNA sequencing with chain terminating inhibitors. *Biotechnology* **24**, 104-108.

Schulte-Merker, S., Ho, R. K., Herrmann, B. G. and Nusslein-Volhard, C. (1992). The protein product of the zebrafish homologue of the mouse *T* gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116**, 1021-1032.

Sherwood, D. R. and McClay, D. R. (2001). LvNotch signaling plays a dual role in regulating the position of the ectoderm-endoderm boundary in the sea urchin embryo. *Development* **128**, 2221-3222.

Shoguchi, E., Satoh, N. and Maruyama, Y. K. (2000). A starfish homolog of mouse *T-brain-1* is expressed in the archenteron of Asterina pectinifera embryos: Possible involvement of two T-box genes in starfish gastrulation. *Dev. Growth Differ*. **42**, 61-68.

Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D. and Herrmann, B. G. (1991). Expression of a *Xenopus* homolog of *Brachyury* (*T*) is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

Smith, J. (1997). *Brachyury* and the T-box genes. *Curr. Opin. Genet. Dev.* **7**, 474-480.

Sweet, H. C., Gehring, M. and Ettensohn, C. A. (2002). LvDelta is a mesoderm-inducing signal in the sea urchin embryo and can endow blastomeres with organizer-like properties. *Development* **129**, 1945-1955.

Sweet, H. C., Hodor, P. G. and Ettensohn, C. A. (1999). The role of micromere signaling in Notch activation and mesoderm specification during sea urchin embryogenesis. *Development* **126**, 5255-5265.

Tagawa, K., Humphreys, T. and Satoh, N. (2000). *T-Brain* expression in the apical organ of hemichordate tornaria larvae suggests its evolutionary link to the vertebrate forebrain. *Mol. Dev. Evol.* **288**, 23-31.

Urry, L. A., Hamilton, P. C., Killian, C. E. and Wilt, F. H. (2000). Expression of spicule matrix proteins in the sea urchin embryo during normal and experimentally altered spiculogenesis. *Dev. Biol.* **225**, 201-213.

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., Brandhorst, B. P. and Klein, W. H. (1995). Autonomous and non-autonomous differentiation of ectoderm in different sea urchin species. *Development* **121**, 1497-1505.

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

Yamasu, K. and Wilt, F. H. (1999). Functional organization of DNA elements regulating *SM30*, a spicule matrix gene of sea urchin embryos. *Dev. Growth Differ*. **41**, 81-91.

Yasuo, H. and Satoh, N. (1994). An ascidian homolog of the mouse *Brachyury* (*T*) gene is expressed exclusively in notochord cells at the fate restricted stage. *Dev. Growth Differ*. **36**, 9-18.

Yonei-Tamura, S., Tamura, K., Tsukui, T. and Izpisua Belmonte, J. C. (1999). Spatially and temporally-restricted expression of two T-box genes during zebrafish embryogenesis. *Mech. Dev*. **80**, 219-221.

Yoshikawa, S. I. (1997). Oral/aboral ectoderm differentiation of the sea urchin embryo depends on a planar or secretory signal from the vegetal hemisphere. *Dev. Growth Differ*. **39**, 319-327.