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Impairing Otp homeodomain function in oral ectoderm cells affects skeletogenesis in sea urchin embryos

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Abstract

In the sea urchin embryo skeletogenesis is the result of a complex series of molecular and cellular events that coordinate the morphogenetic process. Past and recent evidence strongly indicate that skeletal initiation and growth are strictly dependent on signals emanating from the oral ectodermal wall. As previously suggested, Orthopedia (Otp), a homeodomain-containing transcription factor specifically expressed in a small subset of oral ectoderm cells, might be implicated in this signalling pathway. In this study, we utilize three different strategies to address the issue of whether Otp is an upstream regulator of sketelogenesis. We describe the effects of microinjection of Otp morpholino-substituted antisense oligonucleotides and dominant-negative *Otp-engrailed* mRNA in *Paracentrotus lividus* embryos. We demonstrate that inhibition of Otp expression completely abolishes skeletal synthesis. By contrast, coinjection of *Otp* mRNA and the morpholino antisense oligonucleotide specifically rescues the skeletogenic program. In addition, localized ectodermal expression of the *Otp-GFP* fusion gene construct driven by the hatching enzyme promoter, induces ectopic and abnormal spiculogenesis. We further show that an indirect target of this homeoprotein is the skeletogenic specific gene *SM30*, whose expression is known to be under the strict control of the oral ectoderm territory. Based on these results, we conclude that Otp triggers the ectoderm-specific signal that promotes skeletogenesis.

Keywords: *Orthopedia* homeobox; Sea urchin embryo; Skeletogenesis; Morpholino oligonucleotides

Introduction

The synthesis of the skeleton is one of the key events during sea urchin development. Skeletal rods form the framework of the larva and transmit tension to the ectoderm (Gustafson and Wolpert, 1961). With their support the embryonic arms elongate, affecting the ability of the larvae to swim and feed (Giudice, 1973; Pennington and Strathman, 1990).

In the indirect developing sea urchin embryos skeletal synthesis is initiated in the blastocoele at the mid-gastrula stage by the primary mesenchyme cells (PMCs). Descendants of the micromeres, PMCs arise at the 16-cell stage as the founder cells of the skeletogenic lineage. At the blastula stage PMCs enter the blastocoele, migrating and arranging into a ring parallel to the posterior wall of the larva. At the right and left corners of the ventral side of the embryo a large number of PMCs group into two clusters, from which triradiate spicule primordia arise (reviewed by Okazaki, 1975b). If cultured in vitro in the presence of horse serum, isolated micromeres undergo an autonomous program of differentiation (Okazaki, 1975a).

Molecular details of the initial specification of this lineage begin to be highlighted. Inhibition of accumulation of β-catenin, a cofactor of the Tcf/Lef-1 family of transcription factors in the micromere nuclei, strongly represses PMC formation. This indicates that β-catenin is a fundamental component in the molecular pathway that autonomously specifies the micromeres to a skeletogenic fate (Logan et al., 1999). On the other hand, absence or very low levels of SpSoxB1, a Sox family transcription factor, that presents a

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complementary pattern with respect to that of β-catenin (Kenny et al., 1999), appear essential to provide micromeres a unique repertoire of transcriptional regulators (reviewed in Angerer and Angerer, 2003). Although functional assays are not yet available, an important role can be hypothesized for the Otx transcription factor, which transiently translocates in the nuclei of micromeres at the 16-cell stage (Chuang et al., 1996). It has been reported that Pmar1, a homeodomain-containing transcriptional repressor, which is activated only in the micromere population, is indirectly responsible for the activation of at least the ligand *delta* and three regulators, *ets* (Kurokawa et al., 1999), *dri*, and *tbr* (Croce et al., 2001). The expression of these genes is repressed everywhere except in the micromere lineage (Oliveri et al., 2002). As a consequence of these and presumably other events, only micromeres are committed to an irreversible regulatory state that leads to the activation of the differentiation genes of the skeletogenic mesenchyme. This basic program of lineage specification includes the activation of several skeletal-specific genes: *msp130* (Anstrom et al., 1987; Harkey et al., 1992), *Pm27* (Harkey et al., 1995), *SM37* (Lee et al., 1999), and *SM50* (Benson et al., 1987).

Besides the molecular events occurring during early cleavage that lead to the specification of the skeletogenic lineage, old and recent lines of evidence indicate that oral ectoderm patterning determines the position and orientation of the larval skeleton (von Ubish, 1937; Gustafson and Wolpert, 1961; Ettensohn and McClay, 1986; Hardin et al., 1992; Armstrong et al., 1993; Ettensohn and Malinda, 1993; Ettensohn et al., 1997). At mid-gastrula stage, when PMCs cluster into ventrolateral positions, transcription of the skeletogenic gene *SM30* (George et al., 1991), which encodes for the most abundant spicule matrix protein, is activated. The gene plays a key role in skeletal initiation and growth and its expression is strictly dependent on signals emanating from small areas of the oral ectoderm (Guss and Ettensohn, 1997). The dependence of *SM30* synthesis from external cues is also demonstrated by recent experiments with in vitro cultured micromeres. In fact, SM30 protein accumulation is selectively depressed if serum is withdrawn from the culture medium, while the inhibition effect on the accumulation of other proteins is very modest (Urry et al., 2000).

Despite the wealth of information, ectoderm factors involved in the epithelial-mesenchymal signalling have not been identified yet. In a previous study, we suggested that Orthopedia (Otp), a homeodomain-containing transcription factor expressed in a restricted manner in the oral ectoderm territory of *Paracentrotus lividus* embryos, is involved in ectoderm-mesenchymal interaction and its expression influences skeletal patterning (Di Bernardo et al., 1999, 2000). The following evidence support such a role. Transcription of the *Otp* gene starts at mid-gastrula stage, i.e., at the onset of skeletal synthesis (Wilt, 1987; Ettensohn et al., 1997) and expression is restricted in two pairs of oral ectodermal cells of the ventrolateral region. These thickened areas, symmetric with respect to the embryo's left-right axis, are positioned just above the sites where primary mesenchyme cell clusters fuse and secrete skeletal elements. Even at later stages, there is always a strong correspondence between the Otp-expressing cells and the sites of active skeletal growth. The correlation between PMCs aggregates and Otp expression is reinforced by the observation that the vegetalizing agent lithium chloride shifts both the PMCs (Gustafson and Wolpert, 1961) and the Otp-expressing cells toward the animal pole. Moreover, nickel treatment, which is known to alter commitment of ectodermal cells along the oral-aboral axis (Hardin et al., 1992), induces overexpression of the *Otp* gene and the formation of multiple spicule rudiments in the corresponding areas of the blastocoele. Finally, ectopic expression of the Otp regulator causes abnormal skeletal development to occur at multiple sites with an effect similar to that exerted by $NiCl_2$ (Di Bernardo et al., 1999).

Here, by loss of function assays, we demonstrate that Otp acts as a positive regulator in a subset of oral ectodermal cells that transmit short range signals to the underlying mesenchyme. Lack of these signals leads to the development of skeletonless embryos and this effect is either observed by the injection of specific morpholino-substituted antisense oligonucleotides or the expression of a dominant-negative repressor construct. We also show that ectopic skeletal synthesis is rescued by the presence of *Otp* functional mRNA and induced by the expression in the ectoderm of the Otp-GFP fusion protein. Finally, we provide evidence that inhibition of Otp function specifically affects the synthesis of the skeletogenic gene *SM30*.

Materials and methods

Embryo culture and RNA extraction

Adult *Paracentrotus lividus* were obtained from fishermen of the Sicilian coast and maintained in a laboratory tank. Gametes were harvested and eggs fertilized and cultured as previously described (Giudice, 1973). To be microinjected, eggs were dejellied by treatment with acidified Millipore Filtered Sea Water (MFSW) and rapidly brought back to the normal pH value. After microinjection, embryos were raised at a temperature of 18°C until reaching the desired stage.

Microinjection of morpholino-substituted oligonucleotides, synthetic mRNAs, and DNA constructs

Microinjections were performed as follows. Oligonucleotides, synthetic mRNAs, and DNA constructs were resuspended in 30% glycerol and, in selected experiments, Texas Red-conjugated dextrane (Molecular Probes) was added at a concentration of 5%. Morpholino-substituted oligonucleotides were purchased from Gene Tools, Corvallis, OR. Nucleotide sequence of antisense (mASOtp) and invert

(mInOtp) morpholino oligomers were, respectively: 5′GGGCTAATGTTCGTTCCATCCTATC 3′ and 5′ CTATCCTACCTTGCTTGTAATCGGG 3′. mASOtp and mInOtp were resuspended in ultrapure water (Invitrogen) and 2 pl of a 500 μM solution were injected.

A dominant negative construct was obtained by fusing the Otp homeodomain encoding sequences to those of the Engrailed repressor domain cloned in the CS2+nls expression vector. In vitro capped mRNAs were transcribed from both the linearized CS2+nls-En-Otp fusion and CS2+nlsEn (as control) using the Sp6 mMessage mMachine kit (Ambion). Synthetic mRNAs were resuspended in ultrapure RNase-free water at 0.5 mg/ml and 2 pl, corresponding to 1 pg mRNA/egg, were then injected.

In the rescue experiments 3×10^8 molecules of mASOtp or mInOtp were coinjected with 1.5×10^6 molecules of an in vitro transcribed mRNA from the linearized CS2+MT-Otp expression vector (Di Bernardo et al., 1999).

Otp-GFP encoding fusion protein construct was obtained by cloning the Otp coding region downstream of the 2.9 kb of *P. lividus* hatching enzyme (HE) regulatory sequences. The Otp-GFP coding regions were located downstream of the 5′ leader and in frame with the first three codons of the *HE* gene, from which translation is likely to start. The control plasmid (pHE-GFP) was a kind gift of C. Gache.

At the proper stages, embryos were fixed with 4% formaldehyde. DIC, bright-field, or fluorescence images were captured or photographed.

Cloning of SM30 and SM50 P. lividus orthologues

10^5 plaques of a 30 h prism-stage cDNA library were screened with a 0.9-kb *Hin*dIII-*Sac*I fragment from *SM30* (George et al., 1991) and a 1.3-kb *Eco*RI-*Eco*RI fragment obtained from *SM50*, respectively (Sucov et al., 1987). Both cDNAs encoded for *S. purpuratus* SM30 and SM50 proteins. Filters were prehybridized at 60°C in 6× SSC, 5× Denhardt's solution, and 0.5% sodium dodecyl sulfate (SDS). Hybridization was carried out for 16 h in the presence of ^{32}P labelled *SM30* and *SM50* DNA fragments purified from *S. purpuratus* genes. Filters were repeatedly washed at 60°C in 2× and 0.2× SSC containing 0.5% SDS. Plaques were purified and recombinant plasmids were sequenced on both strands using Sequenase sequencing kit (USB).

RNA extraction, reverse transcription–polymerase chain reaction (RT-PCR) and Southern blot hybridization

Total RNA was extracted from 2-day-old embryos injected with mASOtp or mInOtp, using the High Pure RNA Isolation kit (Roche). RNAs from five morpholino AS (Antisense) or In (Invert) injected embryos were reverse-transcribed and amplification reactions were carried out using the Titan One Tube RT-PCR kit (Roche). Oligomers derived from the *P. lividus SM30*, *SM50*, and *MBF-1* nucleotide sequences were used as primers. *PISM30*, *PISM50*, and *MBF-1* oligonucleotide forward primers were 5′ GTGTACCAGATCAACAAGAC 3′, 5′ GATCTGCTGGCAGTCACT 3′, and 5′ GGAATGAAAACACAGAGCAGCCT 3′, respectively. Reverse primers were 5′ GACTTGGTTATTGAACATCTG 3′ for *PISM30*, 5′ TGCGAACACGTCAGTATGT 3′ for *PISM50*, and 5′ CTGGTAGACGATGTTATCCCC 3′ for *MBF-1*. Annealing and extension occurred at 55°C and 68°C, respectively. Aliquots of the amplification products were analysed on a 3% Nusieve agarose gel, blotted onto Nytran 0.45 μm (Schleicher & Schuell), and hybridized with *PISM30*-, *PISM50*-, and *MBF-1*-specific probes. Filters were washed at high stringency and exposed to X-Omat AR (Kodak). Films were scanned using a Chemi Doc (Bio-Rad Laboratories).

Results

Inhibition of Otp translation affects skeletal initiation and growth

To assess the role of the Otp regulator in embryo patterning, we used two different perturbation approaches. First, we injected morpholino antisense (AS) oligonucleotides to block *Otp* translation. Morpholino-substituted oligonucleotides are very stable molecules with limited toxic effects that, by annealing to a specific target sequence, block mRNA translation. The "morpholino" technology has been successfully used to knock out genes in several organisms (for a review, see Heasman, 2002), including sea urchins (Howard et al., 2001; Davidson et al., 2002; Moore et al., 2002; Sweet et al., 2002). We designed a 25-mer antisense oligonucleotide (mASOtp), spanning a region comprised between nucleotide (nt) −6 and +19 of the cDNA (Di Bernardo et al., 1999) and, as a control, a morpholino oligo, whose sequence was inverted with respect to the former (mInOtp). In a preliminary experiment, in vitro transcribed *Otp* mRNA was translated in a rabbit reticulocyte lysate to which antisense or invert morpholino oligomers were added. Analysis of the products by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) clearly demonstrated that translation was specifically inhibited in the sample containing the antisense morpholino oligonucleotide, but occurred normally in the control (not shown).

To test its effect on living embryos, we injected *P. lividus* fertilized eggs with 2 pl of antisense or control Otp morpholino oligonucleotide solution, at concentrations ranging from 100 to 500 μM. Different batches of eggs were injected and embryos were allowed to develop until controls reached the pluteus stage (48 h after fertilization). Careful microscopic observations indicated that injection of mASOtp up to a concentration of 400 μM did not have significant effects on development and the majority of the injected embryos appeared normal (not shown). Because of the highly restricted expression of the *Otp* gene, initially occurring in two pairs of ectoderm cells (Di Bernardo et al.,

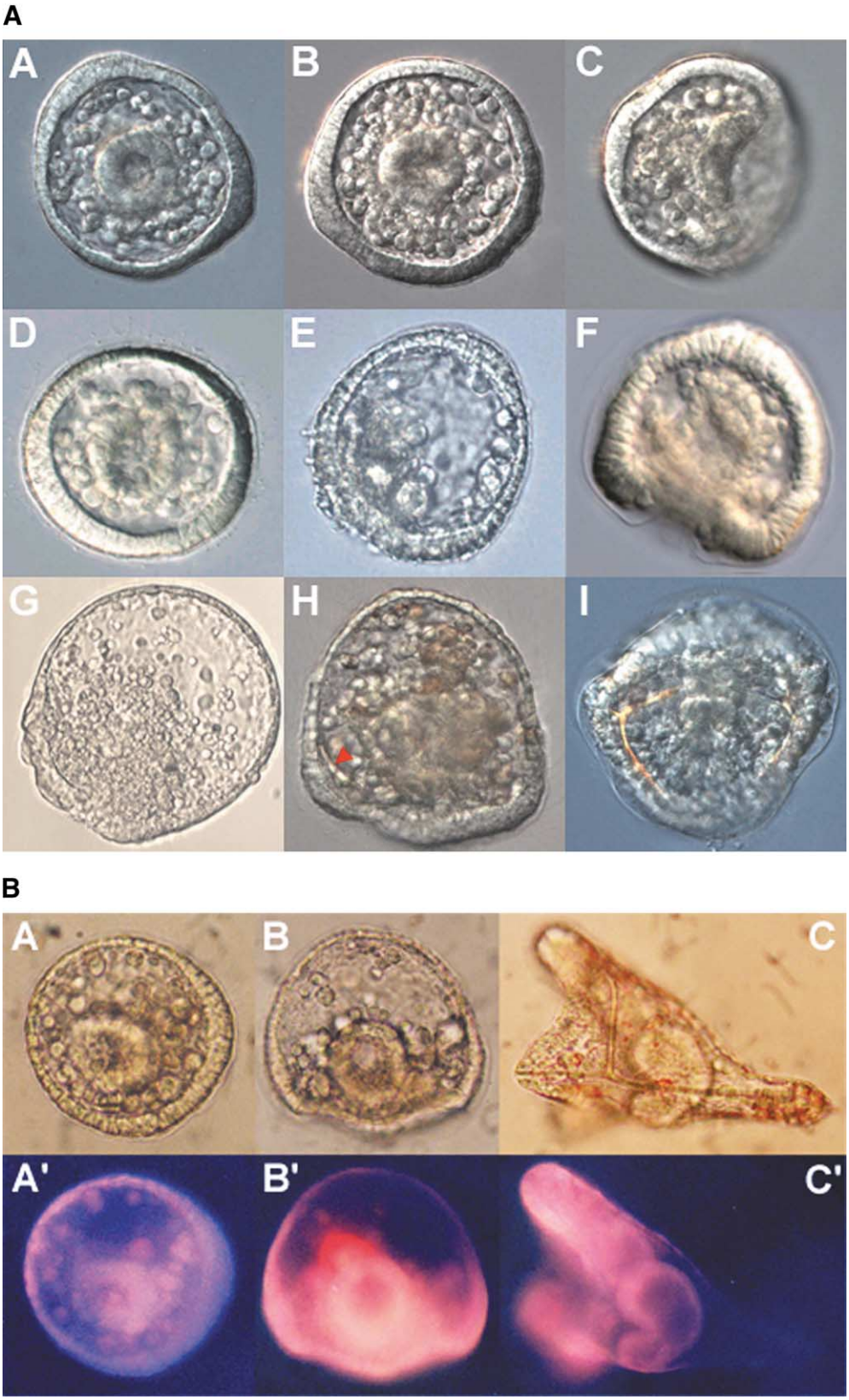
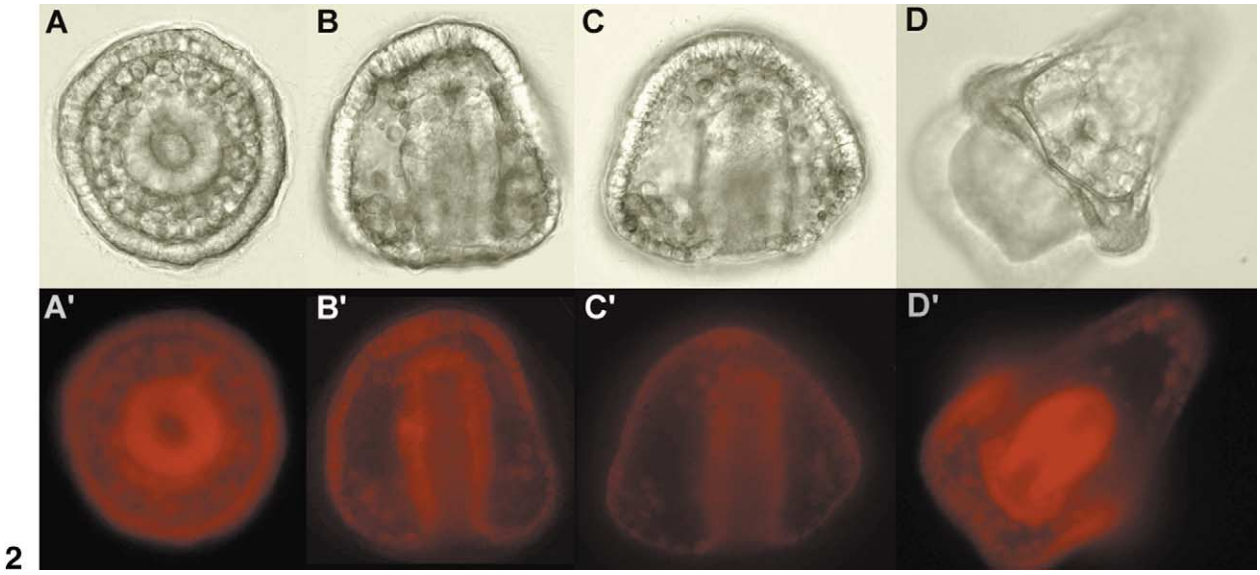


Fig. 1. (A) Injection of *Otp* morpholino substituted oligonucleotides and effects on *P. lividus* development. Two picoliters of 500 μM mASOtp (A–H) was injected into fertilized eggs and embryos observed after 26 h (A–C), 42 h (D and E), and 48 h (F–H) of development. All embryos lack any skeletal element except the embryo in H, where a red arrowhead points to a single spicule element formed on one side. A 26 h embryo injected with the same amount of mInOtp is shown in I. A, B, D, and I show embryos viewed along the animal vegetal axis, C and E–G are vegetal-lateral and lateral images, respectively. (B) Bright-field and fluorescence images of 48-h-old embryos coinjected with mASOtp (A, A′, B, B′) or mInOtp (C, C′) and dextran-conjugated Texas Red. Regular diffusion of the dye and the morpholino oligomers occurred in all embryos, but only those injected with the control morpholino oligonucleotide were able to synthesize skeleton.



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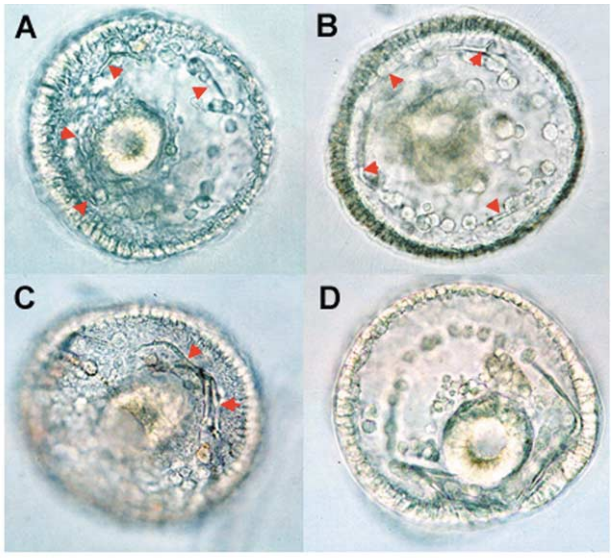

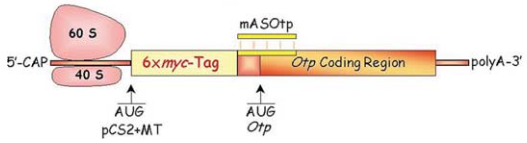

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Fig. 2. Injection of a synthetic mRNA encoding for Otp as dominant repressor specifically blocks skeletal synthesis. CS2+nls-En-Otp consists in a fusion between the engrailed repressor domain and the Otp coding region in the CS2+nls expression vector. One picogram of the chimeric RNA was injected in *P. lividus* eggs and embryos were observed after 48 h of development under bright-field conditions. (A) An embryo viewed along the animal-vegetal axis. (B and C) Lateral view of two embryos at the same stage. They show an irregular distribution of the PMCs (primary mesenchyme cells) in the blastocoele and lack of skeleton. Note that the embryos, as repeatedly observed, do not elongate arms. (D) A 48-h pluteus stage embryo injected with the same amount of mRNA encoding for the En repressor (CS2+nls-En).

Fig. 3. Otp expression in morpholino AS (antisense) oligonucleotide-injected embryos rescues skeletogenesis. *P. lividus* eggs were coinjected with synthetic capped Otp-mRNA transcribed from CS2+MTOtp construct and mASOtp (molecular ratio 1:200). As schematically shown in the drawing below the photographs, the Otp protein coding sequence is fused to the myc-tag epitope. Translation of this chimeric mRNA starts at the first myc-tag AUG codon and mASOtp, annealed to the specific *Otp* target sequence, is removed by the translocation of the ribosome during translation. (A–D) Embryos are photographed at 48 h of development. (A and B) Bright-field images of embryos showing multiple spicules radially placed in the blastocoele, as indicated by arrowheads. (C) Image of an embryo that displays irregular skeletal elements with several branches, pointed by two red arrowheads. (D) Embryo showing symmetric spicules, more similar to those developed within a normal embryo.

1999), we reasoned that inhibition of translation could only be attained by the injection of a higher concentration of the AS oligonucleotide. Indeed, as it is shown below, injection of 500 μM mASOtp oligo produced reproducible altered phenotypes in about 70–75% of embryos. Only 6–10% of embryos were not affected. In contrast, a normal phenotype was observed in more than 80% of embryos injected with 500 μM of mInOtp oligonucleotide. In all experiments and with both types of oligonucleotides, 10–20% of the embryos degenerated. As shown in Fig. 1A, mASOtp injected embryos at 26 h (A–C), 42 h (D and E), and 48 h (F–H) of development clearly presented dramatic changes in their morphology. Lack of skeletal elements and an irregular distribution of PMCs in the blastocoele were the main effects of antisense-mediated Otp block of translation. The number of PMCs did not seem to be reduced with respect to the controls, but their arrangement was strongly altered. All embryos were viable and swam regularly, but none of them, even after 48 h of development, displayed the distinctive angular shape of the pluteus larvae. The embryo's body, which is normally supported by the long skeletal rods, appeared rounded and displayed a radial distribution of PMCs. Some embryos (about 30%) presented intermediate phenotypes with only one spicule element forming on one side of the embryo, as indicated by the red arrowhead in Fig. 1H, but these spicules never elongate. One of the most recurrent effects that we observed after mASOtp oligo microinjection was a defective process in gastrulation. Primary phase of the invagination of the archenteron occurred quite normally, but secondary invagination, which is always associated with the formation of pseudopods at the archenterons tip (Gustafson and Wolpert, 1963), often failed. However, for the reasons that are outlined below, we believe that alteration of the gastrulation process was not likely due to the direct effect of Otp loss of function, but rather to some unspecific perturbation. In fact, in some cases we observed quite normal gastrulae that showed an apparent typical distribution of the primary mesenchyme cells, but again they did not synthesize skeletal elements (Fig. 1F). Fig. 1I shows a normally developed 26-h embryo injected with 500 μM of mInOtp oligonucleotide.

In another series of experiments we coinjected either mASOtp or mInOtp oligonucleotide and Texas Red-conjugated dextran. The majority of the embryos, observed at 48 h of development, showed diffused fluorescence (Fig. 1B), indicating that both dye and morpholino oligonucleotides were uniformly distributed. With this approach we were able to discriminate among the normally developed embryos injected with mASOtp, those that escaped injection (not fluorescent) from those stained embryos in which morpholino did not appear to interfere with *Otp* translation. As expected, fluorescent embryos injected with mASOtp (A, A′, and B, B′) showed skeletal defects and irregular PMCs patterning, while mInOtp-injected embryos developed as normal plutei (C, C′).

Competition of Otp transactivation function inhibits skelotogenesis

To confirm the role played by the Otp regulator in the initiation of skeletogenesis, we made a dominant repressor construct (CS2nls-En-Otp) in which the Otp homeodomain was fused to the *Drosophila* engrailed repressor domain. Another construct, containing only the engrailed repressor encoding part of the protein (CS2nls-En) was used as a control. Chimeric capped mRNAs were transcribed in vitro and 1 pg of each mRNA was injected in fertilized *P. lividus* eggs. The effects are shown in Fig. 2; 48-h-old embryos, representative of a series of experiments and viewed respectively from the vegetal side (A) and lateral perspective (B and C), showed that the main action of Otp as a forced repressor was that to prevent the formation of embryo skeleton. PMCs entered the blastocoele at the right time, migrating in the embryo cavity, but they were never able to form spicules. Conversely, embryos injected with the same amount of the control construct CS2-nls-En or with glycerol developed normally (D). To monitor injection, embryos were coinjected with the two constructs and Texas Red-conjugated dextran, indicating that inhibition of skeletogenesis was due to the injection of the dominant-negative construct (Fig. 2A′–D′). From these experiments we conclude that the inhibition of Otp function blocks the activation of the skeletogenic program by the primary mesenchyme cells.

Rescue of Otp function restores skeletogenesis

Previous studies showed that Otp gain of function and its expression in ectopic positions caused dramatic alterations in skeletal patterning, such as the formation of supernumerary triradiate spicule elements and the development of abnormal rods (Di Bernardo et al., 1999). Based on this evidence, we addressed the question of whether or not the expression of the Otp transcription factor was sufficient and necessary to reactivate ectoderm to mesoderm signalling and to restore skeletal morphogenesis in morpholino-perturbed embryos. To avoid that the morpholino AS oligomer inhibits translation of the exogenous mRNA, we utilized an in-frame fusion of the myc epitope to the Otp protein coding region (drawing of Fig. 3). As previously shown, such a chimeric mRNA is efficiently translated in *P. lividus* embryo (Di Bernardo et al., 1999). Because morpholino oligomers are effective only when designed on sequences that lie around mRNA initiation of translation (Summerton and Weller, 1997), we expected the translation of the chimeric *Myc-Otp* mRNA to not be inhibited by the mASOtp. Indeed the results presented in Fig. 3 fulfilled this assumption. The same batch of eggs was then divided in different aliquots. One aliquot was microinjected with 500 μM mASOtp and another was coinjected with the same amount of mASOtp and 1 pg of in vitro transcribed CS2+MTOtp-capped mRNA. The molecular ratio of mASOtp to CS2+MTOtp was 200 to 1. As a control, fertilized eggs were injected

either with 1 pg of the functional *Otp* mRNA or 30% glycerol. Results were as follows: mASOtp-injected embryos displayed the expected phenotypes, lacking skeletal elements, while glycerol-injected embryos developed normally (not shown). After 48 h of development embryos injected with *Otp* mRNA exhibited a remarkably abnormal skeletogenesis with supernumerary spicules and, as development proceeded, irregularly patterned skeletal rods. Because these results were identical to those previously obtained (Di Bernardo et al., 1999) they are not shown here. Representative examples of embryos coinjected with mASOtp oligonucleotide and synthetic *myc-Otp* mRNA, are shown in Fig. 3. Embryos, observed after 48 h of development, clearly exhibited a renewed ability to make skeleton, although their pattern was highly perturbed. Embryos in (A) and (B) reveal a radialized PMCs arrangement and multiple spicule elements quite regularly spaced in the blastocoele. These rods, which differ in length and shape, are pointed out by red arrowheads. The embryo in C shows the development of abnormal spicules with irregular side branches, similar to those previously obtained in Otp mis-expression experiments (Di Bernardo et al., 1999). The embryo in D displays a certain bilateral symmetry that more resembles that of a normally developed embryo. In all cases, although at a different extent and with variable patterns, *myc-Otp* mRNA injection rescued the ability of embryos to synthesize embryonic skeleton. Only embryos coinjected with functional *Otp* mRNA were able to develop skeletal elements de novo. Nevertheless, as we observed in a number of experiments, embryos were not able to acquire the predicted shape of pluteus larvae at the end of embryogenesis.

Localized expression of the Otp gene is able to induce the formation of extra skeletal elements

To obtain further insights on the relationship between Otp expression and skeletogenesis, we assessed whether clonal expression of the *Otp* gene can lead to the appearance of extra skeletal rods or spicule primordia. To do this, we injected a *HE-Otp-GFP* DNA construct, whose expression was placed under the control of the 2.9-kb regulatory region of the *P. lividus* hatching enzyme gene. The promoter fragment is sufficient to drive the correct expression in an ectoderm-specific manner (Ghiglione et al., 1997). This construct or the control lacking Otp (*HE-GFP*) were coinjected in fertilized *P. lividus* eggs with Texas Red-conjugated dextrane and the embryos were observed after 20 and 46 h of development. As expected, green fluorescence was specifically detected in the ectoderm (Fig. 4A). Whereas *HE-GFP* expression occurred in a high number of ectodermal cells at both developmental stages (Fig. 4A, and not shown), scattered and less fluorescent cells were observed in *HE-Otp-GFP*-injected embryos (Fig. 4B, G, J, and K). This evidence and the fact that the number of Otp-GFP-stained cells decreased as development proceeded (not shown) most likely indicate a low stability of the chimeric protein.

To obtain statistically relevant results we scored thousands of injected embryos. Fig. 4 shows some examples that depict the variable repertoire of embryonic phenotypes and skeletal patterns. B and C respectively represent fluorescent and bright-field images of a 46-h pluteus stage embryo showing expression of the HE-Otp-GFP fusion protein in a small number of ectodermal cells (B). Close inspection of the embryo's skeleton in the bright-field image clearly revealed the presence of an extra triradiate spicule in correspondence of the fluorescent cells (C). In the embryo shown in Fig. 4D–G, expression of the exogenous construct occurred in a number of cells scattered at different ectodermal locations. Although invagination of the archenteron occurred normally and the mouth opened at the right place, skeletal aspects of morphogenesis were highly altered. Rods were irregularly patterned and had lost their usual organization. Different focal planes show the complicated arrangement of the skeleton coupled with a random orientation and distribution of the fluorescent cells. Another mispatterned embryo is shown in H–J. Here, again, arrangement of the skeletal elements along the oral-aboral axis is highly irregular. As well as shown in the previous image, the shape of the embryo appeared rounded and not modelled by the elongating skeleton, while other embryos showed a much more localized effect and a normal shape. In this last category we can include those transgenic embryos in which relation of cause and effect is difficult to interpret (e.g., the formation of extremely small spicules in correspondence of one or two Otp-expressing cells). In the last image (K and L), morphology and development appear normal in a 46-h-old embryo. Nevertheless, in correspondence to just four neighbouring cells of the oral ectoderm territory expressing the *Otp* construct, we could see a small skeletal extrusion emerging from one anterolateral rod. This extra branch, more evident in the enlargement (M), is indicated by a blue arrow. In this embryo, Otp ectopic expression probably interfered with the normal branching of the growing skeleton.

Otp is required for the expression of the SM30 skeletogenic gene

Experiments described in the previous sections strongly suggest that the expression of Otp induces the underlying mesenchyme to initiate skeletogenesis. Otp should thus be involved in the pathway that leads to the activation of the *SM30* gene that initiates skeletogenesis in PMCs and whose expression is strictly dependent on ectodermal cues (Guss and Ettensohn, 1997). Conversely, we expect that the expression of other skeletal specific genes should be independent from Otp function as well as that of unrelated genes. To address these questions, we isolated the *SM30* and *SM50* genes from a *P. lividus* prism stage cDNA library by using *S. purpuratus* cDNA probes (kind gifts of F. Wilt). Se-

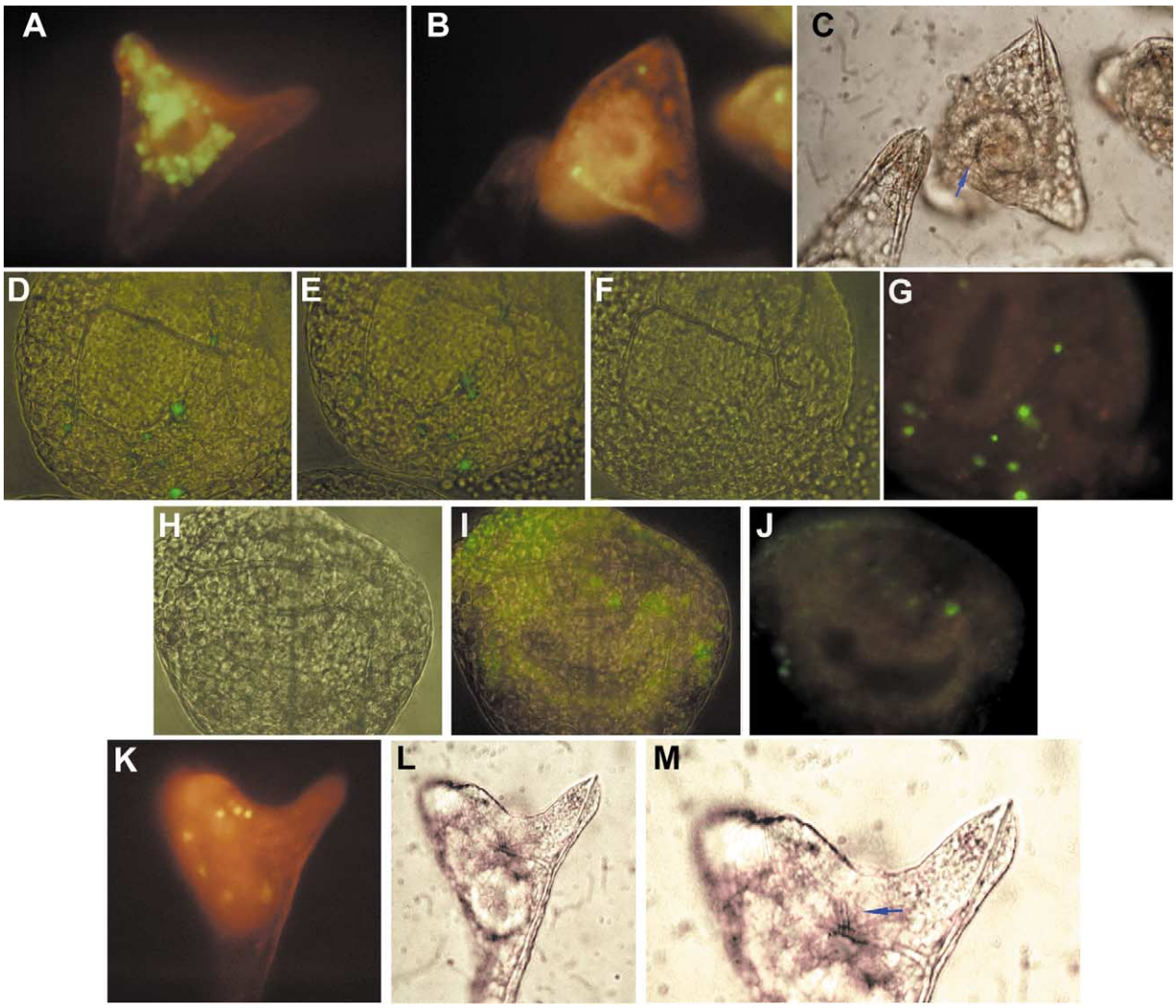

Fig. 4. Expression of the *Otp* transgene induces the formation of extra skeletal elements. *P. lividus*-fertilized eggs were injected with 0.4 pg of *HE-GFP* (A) or *HE-OTP-GFP* DNA constructs (B–M) and photographed after 46 h of development. The number of fluorescent cells is higher in the control (A) than in the embryo injected with the fusion construct (B). A triradiate extra spicule that forms in the area underlying the Otp expressing cells is indicated by a blue arrow in the corresponding bright-field image (C). (D–F) Merged images of different focal planes of an embryo showing the position of the green fluorescent cells and the formation of irregularly patterned skeletal rods. (G) The same embryo photographed under fluorescence illumination. (H–J) Bright-field, merged and fluorescence images, respectively, show the expression of the *Otp* transgene and the abnormal skeletal organization in another embryo. (K and L) Fluorescence and bright-field images of a pluteus stage embryo. By the injection of *HE-OTP-GFP* construct, expression occurred in a limited number of cells. Careful observation of the skeletal pattern pointed out the growth of an extra branch in the anterolateral rod that is indicated by an arrow in the enlarged image (M).

quence determination and comparison to the *SM30* and *SM50* genes of other sea urchin species confirmed that the *P. lividus* genes were the orthologues of the *S. purpuratus* counterparts and other sea urchin species (Fig. 5). PMCs specific expression of the *PISM30* and *PISM50* was demonstrated by whole mount in situ hybridization (not shown).

Embryos were injected with 500 μM mASOtp or mInOtp oligonucleotides and after 48 h of development, total RNA from five perturbed embryos was reverse-transcribed and coamplified with specific oligonucleotides designed on *SM50* and *SM30 P. lividus* genes. Aliquots of the amplified DNA were withdrawn at 15, 20, and 23 cycles, then blotted and hybridized to *PISM50*- and *PISM30*-specific probes. Results of such a hybridization are shown in Fig. 6. They clearly show that morpholino antisense oligonucleotide injections greatly reduce the expression of the skeletogenic gene *SM30*, which is tightly coupled to the deposition of the biomineralized spicules and dependent on ectodermal signals (Wilt, 1997). However, they do not influence the continuous expression of *SM50*, even at late developmental stages. To normalize with a transcript whose expression is likely to be unaffected by Otp-specific perturbation, the same amount of RNA extracted from perturbed or control embryos was coamplified with specific oligonucleotides respectively corresponding to *P. lividus SM30* and modulator binding factor (*MBF-1*) genes. *MBF-1*, encoding

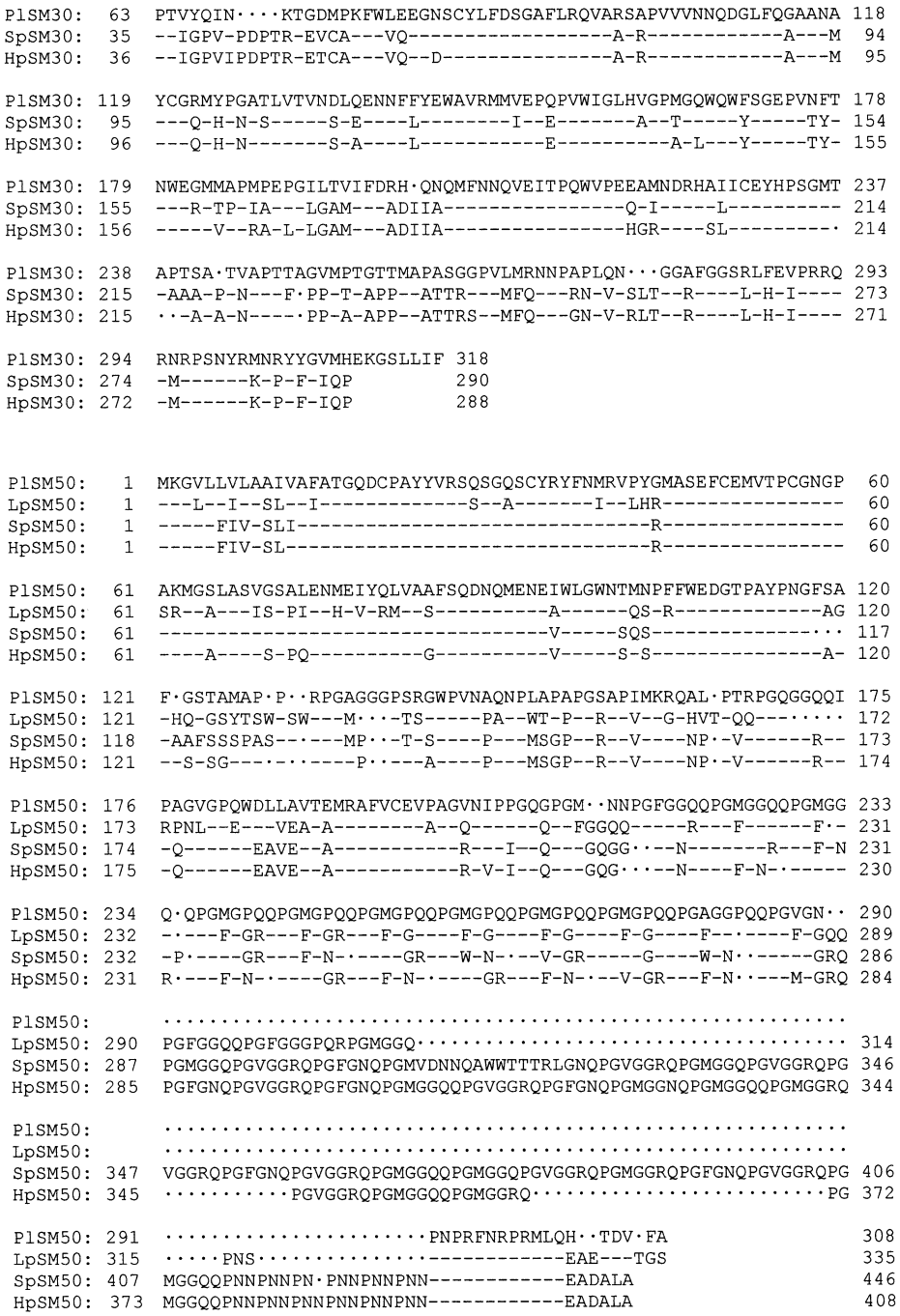

Fig. 5. Comparison of the deduced amino acid sequences among *P. lividus SM30* and *SM50* genes and orthologues isolated from *S. purpuratus* and *H. pulcherrimus* and for *SM50* also from *L. pictus. PISM30* sequence is not complete at the NH_2 terminus. Identities with *S. purpuratus* proteins were 64% and 70%, respectively, for *PISM30* and *PISM50.*

for a Zn finger enhancer binding protein of the sea urchin *α-H2A* histone gene, is constitutively expressed in the egg and at all embryonic developmental stages (Alessandro et al., 2002). Aliquots of the amplification reactions are drawn after 20 and 23 cycles, blotted, and hybridized as already described. Results shown in Fig. 6 confirm that morpholino antisense Otp injection strongly inhibits *SM30* expression, but does not influence the expression of *MBF-1*.

Discussion

The spatially restricted expression of the *Otp* gene in two pairs of ectoderm cells and its expression in ectopic position shown previously, already suggested that Otp is likely to play an important role in the transmission of signals from the ectoderm to primary mesenchyme cells (Di Bernardo et al., 1999, 2000). The results presented in this study dem-

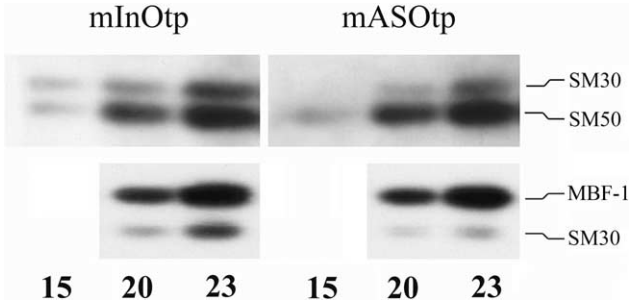

Fig. 6. Downregulation of *SM30* gene transcription by *PlOtp* knock-out. (A) mInOtp or mASOtp were injected into fertilized *P. lividus* eggs and reverse transcription–polymerase chain reaction was carried out on RNAs extracted from embryos injected with the control (left) or the antisense oligomer (right). *SM30*-, *SM50*-, or *MBF-1*-specific oligonucleotides were used to coamplify the respective cDNAs and aliquots of the samples were analysed after 15 and/or 20 and 23 cycles by Southern blot hybridization. Downregulation of *SM30* occurs only in embryos injected with mASOtp. By contrast, the expression of *SM50* or *MBF-1* genes is indistinguishable in embryos injected with either morpholino oligonucleotides.

onstrate that indeed the synthesis of the Otp transcriptional regulator is an essential condition to give PMCs the key input to initiate skeletogenesis in sea urchin embryos.

The skeletogenic mesenchyme descends from the division of the four micromeres that arise at the vegetal pole of the 16-cell stage embryo. Micromeres are involved in at least three different developmental programs, all of which are essential to pattern the embryo. They function as a strong vegetal signalling center to specify the veg2 layer of cells either as endodermal or mesodermal precursors (Ransick and Davidson, 1993; Sherwood and McClay, 1999; McClay et al., 2000; Sweet et al., 1999; Ettensohn and Sweet, 2000; Davidson, 2001). Nevertheless, micromeres have been first characterized as the precursor cells of the skeletogenic mesenchime, whose initial states of determination appear to be largely due to the activation of autonomous programs of gene expression. Later in development, the synthesis and patterning of the embryonic skeleton strictly depend on signals emanating from the ectodermal wall with which primary mesenchime cells make intimate contacts (Gustafson and Wolpert, 1963; Hardin et al., 1992; Armstrong et al., 1993; Ettensohn and Malinda, 1993; Ettensohn et al., 1997; Guss and Ettensohn, 1997; Di Bernardo et al., 1999). These signals must be precise in time and location. Based on our results we strongly suggest that the Otp transcription factor is a localized regulator for skeletal synthesis. In fact, inhibition of Otp function either by the use of a specific morpholino antisense oligonucleotide or through the expression of an En-Otp fusion impairs skeletogenesis and interferes with the regular migration of the PMCs. In the perturbed embryos these cells appear to be randomly arranged. This suggests that *Otp* expression directly influences PMCs distribution and skeletogenesis probably affecting oral ectodermal patterning. The effects respectively observed in embryos that overexpress the gene or a dominant negative form indicate that *Otp* encodes for a transcriptional activator. Indirect evidence comes from experiments showing that *Drosophila* and mouse Otp are able to *trans*-activate a reporter construct carrying the *np* sequence, a binding site consensus found in the *engrailed* regulatory region. The *trans*-activation domain, identified as a small region located downstream the homeodomain, is highly conserved also in sea urchins (Simeone et al., 1994).

The specificity of action of the Otp regulator is shown in this study by mRNA injection effects in the perturbed embryos. We showed that Otp expression is necessary and sufficient to reactivate the skeletogenic program, although these embryos display altered phenotypes. Effects of rescue fully mimic those due to the mis-expression of the gene (Di Bernardo et al., 1999). In both cases highly irregular rods were formed. This pattern is predictable if we take into account that, in normal embryos, *Otp* expression is highly localized (Di Bernardo et al., 1999) and PMCs are constrained by the environment to limit number and size of spicules (Armstrong et al., 1993). Moreover, as with the mis-expression experiments, rescued embryos developed without elongation of the embryonic arms, a process that is known to be the result of mutual interactions between PMCs and ectoderm and requires the integrity of both cell types (Gustafson and Wolpert, 1963; Ettensohn and Malinda, 1993).

Further evidence for a positive role of Otp in the skeletogenic process were gained by clonal expression in the ectoderm of the *Otp-GFP* fusion gene placed under the control of the *HE* promoter (Ghiglione et al., 1997). Nevertheless, aberrant spiculogenesis or an extra triradiate spicule were observed only in a small fraction of injected embryos. Such a low number of affected transgenic embryos is not surprising. First, the Otp-GFP fusion protein is much less stable than the single proteins alone (Fig. 4 and Di Bernardo et al., 1999). More importantly, we believe that the expression of the *Otp-GFP* transgene can be effective only when it takes place in specific ectodermal cells and not in any cell. Hence, appropriate localization of the transgenes is statistically unfavourable. Several lines of evidence are in favour of this hypothesis. As reported in a previous study, the expression of the *Otp* gene was detected immediately before the beginning of spiculogenesis in a pair of symmetric oral ectoderm cells. Increasing the number of *Otp*-expressing cells by $NiCl_2$ treatment did not cause a proportional increase in the number of spicule primordia (Di Bernardo et al., 1999). Consistent with these evidence, we also reported that mis-expression of *Otp* by mRNA injection in a very high number of cells gave rise to the uniform distribution of the protein in the embryo and at most the formation of six foci of spicules (Di Bernardo et al., 1999). Molecular mechanisms by which differences in responsiveness of ectodermal cells are determined are not known. It is likely that Otp requires the presence of colocalized partners, whose function is essential for the activation of the ectoderm to mesoderm signalling pathways. These signals are

specific and directed to PMCs, the only cell type where skeleton is synthesized.

The development of the skeletal system involves the expression of PMC-specific genes whose transcription is temporally and spatially regulated throughout embryogenesis. Analysis of the expression patterns of the *SM30* and *SM50* genes, major constituents of the embryonic skeleton, suggested that the two genes are subjected to different mechanisms of regulation. Several lines of evidence indicated that the *SM50* gene activation is not dependent on external cues to be expressed (Kitajima et al., 1996; Ettensohn et al., 1997), while *SM30* gene uniquely responds to the local control of the ectodermal epithelium (Guss and Ettensohn, 1997; Ettensohn et al., 1997). Furthermore, high levels of *SM30* but not of *SM50* transcripts are directly correlated to the formation of spicule primordia (Guss and Ettensohn, 1997). Thus, we hypothesized that Otp expression is part of the complex signalling network that specifically activates *SM30* gene transcription. Results presented here are in agreement with this assumption. Activation of *SM30* expression is not permitted when we injected Otp inhibitors of function and, as predicted, PMCs did not form spicules. Our results also show that inhibition of Otp function does not have any effect on the expression of the skeletogenic *SM50* gene and the unrelated *MBF-1* (Alessandro et al., 2002). *SM50* transcripts, first detected at low levels in late cleavage embryos (Killian and Wilt, 1989), are present in all PMCs by the mesenchyme blastula stage, long before the activation of the *Otp* gene. *SM50* is nevertheless subjected to some ectodermal gene regulation later in development (Ettensohn et al., 1997). Effects of *PlOtp* on *SM30* are, obviously, indirect. The two genes are expressed in two different cell types that are known to influence each other. The interaction must occur through intermediate factors involving the action of the extracellular matrix (ECM) components. In fact, inhibitors of collagen processing inhibit *SM30*, but not *SM50*, gene expression (Ettensohn et al., 1997).

The list of the genes involved in the regulative network responsible for ectoderm-to-mesoderm signalling is so far incomplete. To date, identification of Otp regulators, partners, and other gene products responsible for the transduction of the proper signals is one of the most important goals that would elucidate the molecular events that control PMCs patterning and sketelogenesis.

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