Int. J. Dev. Biol. 44: 637-643 (2000)

Homeobox genes and sea urchin development

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ABSTRACT We describe the expression of three *Paracentrotus lividus* homeobox-containing genes of the dispersed class during sea urchin embryogenesis and discuss their possible roles in the mechanisms of cell specification and embryo morphogenesis. *PlHbox12* represents the first regulator identified in sea urchin that belongs to the zygotic class of transcription factors. Its early and transient expression and the localization of transcripts suggests that *PlHbox12* is involved in cell fate specification of the oral or aboral ectodermal territories at the early cleavage stages. *PlHbox9* is expressed just after the completion of gastrulation in a narrow stripe of cells at the ectoderm-endoderm boundary. It probably organizes a novel spatial boundary which definitely separates the archenteron and the aboral ectoderm. Finally, the spatial and temporal expression of the *PlOtp* gene strongly indicate that this regulator is conditionally activated in few cells of the oral ectoderm and is involved in patterning of this territory at late stages. Furthermore, our data indicate that *PlOtp* acts upstream of signaling systems that lead to the activation of the primary mesenchyme cell gene expression program and skeletal morphogenesis.

KEY WORDS: *Sea urchin, homeobox genes, PlHbox12, PlHbox9, PlOtp, Hox genes.*

Introduction

It is well known that the homeobox, a 180 bp long DNA segment, is common to a large number of genes isolated from a great variety of organisms (reviewed in: Gehring, 1987; Scott *et al.,* 1989). These genes encode for transcription factors that control cell identity and fate (Affolter *et al.*, 1990; McGinnis and Krumlauf, 1992; Gehring *et al.,*1994; Akam, 1995).

Homeobox containing genes can be classified into two superclasses: the Complex Superclass which encloses all the genes clustered in the homeotic complexes, the *HOM/Hox* complexes, and the Dispersed Superclass whose members are scattered in the genome (Gehring *et al.,*1994). The former, first characterized in *Drosophila* and mammals, displays the striking feature of colinearity: the genes in the complex are expressed in space and time as they are arranged along the chromosome. Genes at the 3' are expressed anteriorly and early, while genes at the 5' are activated towards the more distal part of the body and late in development. *Hox* gene clusters have been characterized in a relatively small number of organisms representative of different taxa (Finnerty and Martindale, 1998). The comparative study of the organization and function of this class of developmental regulatory genes is helping to rewrite the history of the evolution of developmental systems (Purugganan, 1998).

This review deals with homeobox genes of sea urchin, focusing on those expressed in the *Paracentrotus lividus* embryo, that we have recently cloned. We will describe their spatial pattern of expression and discuss their possible role in cell specification and morphogenesis.

Cell specification and gene regulation in the sea urchin embryo

Cell specification in the sea urchin embryo has been extensively described in an exhaustive series of reviews (Davidson, 1989; 1990; 1991; Davidson *et al.*, 1998). The invariant cleavage of the sea urchin embryo generates at the 60 cell stage (sixth division) five polyclonal lineage elements whose specification relies on both the presence of localized determinants and short-range inductive interactions among blastomeres. Cell specification occurs when specific sets of genes are expressed in particular cell types. From lineage tracing experiments, it has been established that blastomeres are already specified by the sixth division as an effect

Abbreviations used in this paper: PMCs, primary mesenchyme cells; A-V, animal-vegetal axis; BMP-1, bone morphogenetic protein-1; O-A, oral-aboral axis; ECM, extracellular matrix.

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0214-6282/2000/$20.00

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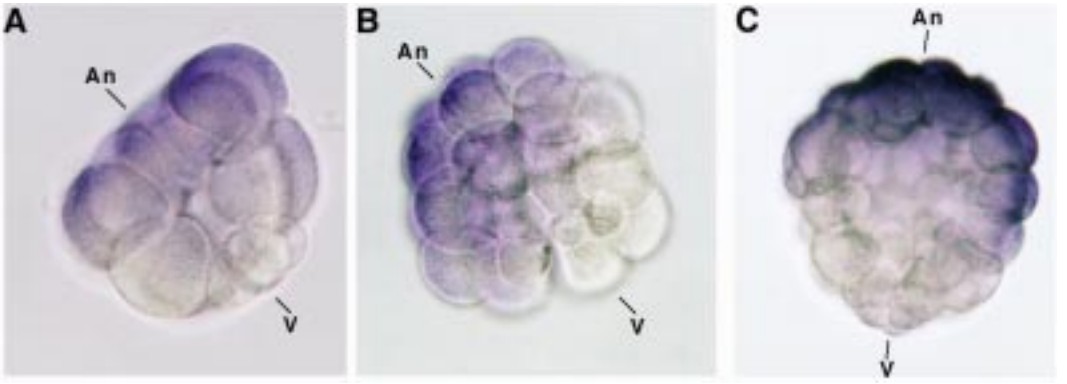

Fig. 1. Spatial expression pattern of the *PlHBox12* gene in *P.lividus* embryos. *In situ hybridization to whole mount embryos with a DIG-UTP labelled antisense RNA.* **(A)** *Lateral view of a 16-cell stage embryo. mRNAs are restricted to some mesomeres and two of the four macromeres.* **(B)** *Lateral-vegetal view of an embryo at 32 cell-stage.* **(C)** *Lateral view of a 64-cell stage embryo. All embryos show the highly asymmetryc expression of the gene. At all stages transcripts are absent from the micromeres at the vegetal pole of the embryos. Animal (An) and vegetal (V) poles are indicated.*

of the invariant cell lineage. This is true for the skeletogenic founder cells and the vegetal plate territory, although commitment occurs later in development. By contrast, the veg1 domain and the ectodermal regions that will give rise to the endoderm-ectoderm and oral-aboral boundaries, will be specified at later developmental stages with lineage independent mechanisms (for a review, see Davidson *et al.*, 1998).

The main role of transcription factors in the specification mechanisms is to create embryo domains in which specific genes have to be turned on or off. Thus, maternal and zygotic transcription factors allow the localized expression of genes in different regions of the embryo, long before any signal of differentiation is displayed. For instance, asymmetries of active transcriptional regulators along the animal-vegetal axis (A-V) (Kenny *et al.,* 1999; Wei *et al.,* 1999), seem to restrict the expression domain of genes encoding for the hatching enzyme and for a metallo-protease protein, similar to BMP-1 and tolloid, in the non vegetal blastomeres of the blastula (Lepage *et al.*,1992 a, b; Reynolds *et al.*, 1992; Wei *et al.*, 1995; Kozlowski *et al.*, 1996). Furthermore SM5O and msp130 genes, whose products contribute to skeletal building and CyIIa, encoding for a cytoskeletal actin, are transcribed in the most vegetal region, (Benson *et al.*, 1987; Anstrom *et al.,* 1987; Guss and Ettensohn, 1997; Arnone *et al.,* 1998), probably under the control of localized activators (Makabe *et al.,* 1995; Arnone *et al.,* 1998; Davidson, 1999).

Spatially restricted gene expression is also observed along the oral-aboral axis (O-A). Unlike the A/V axis, which is established during the oogenesis, the O-A axis is set up by the two cell stage (Cameron *et al.*, 1989). However, as shown by the effects of nickel treatment, it becomes committed by the onset of gastrulation (Hardin *et al.*, 1992) and its position, with respect to the first cleavage, varies from specie to specie (Jeffery, 1992). Aboral and oral specific markers are detectable from late cleavage onward and accumulate in embryos at late stages (Carpenter *et al.,* 1984; Cox *et al.,* 1986; Hardin *et al.,* 1988; Akasaka *et al.,* 1990; Coffman and McClay, 1990). Studies on the cis regulatory regions of some of these genes (Kirckhamer and Davidson, 1996; Gan *et al.,* 1995; Koike *et al.,* 1998) and on their transcriptional binding activities (Kozlowski *et al.,* 1991; Gan and Klein, 1993; Wang *et al.,* 1995; Coffman *et al.,* 1996; Sakamoto *et al.,* 1997), led to the suggestion that correct temporal and spatial patterns of expression in different regions of the early embryo, are due to the activation of positive and negative regulators acting under the control of signaling pathways.

Among the factors controlling gene expression in the sea urchin embryo system, homeobox containing genes are used for different purposes at early and late stages, showing once more that significant differences in the expression patterns of related genes in different systems, are to be considered as models of evolutionary diversification.

The Hox *complex in Echinoderms*

Echinoderms represent the sister group of chordates within deuterostomes. Sea urchins use two different modes of development. Most of the species grow through an indirect larval form, inside of which the definitive adult body develops. Other species directly give rise to the juvenile form. In the former, a group of set-aside cells on the left coelomic sac and the vestibular ectoderm on the surface of the larva, undergo complex morphogenetic processes that *de novo* build the rudiment of the adult body (reviewed in Davidson *et al.,* 1998). After metamorphosis, likewise the direct developing sea urchins, they display an evident pentameric pattern, with no reminiscence of the larval bilateral symmetry. Despite these features, unique in the animal kingdom, a single *Hox* gene cluster has been identified and anatomically dissected in both the direct *H. erythrogramma* and the indirect developing *S. purpuratus*. species (Popodi *et al.,*1996; Martinez *et al.,* 1999). Expression studies of the ten genes of the *S. purpuratus Hox* cluster revealed that only two of them are activated during larval development and that they are mostly utilized during the construction of the complex

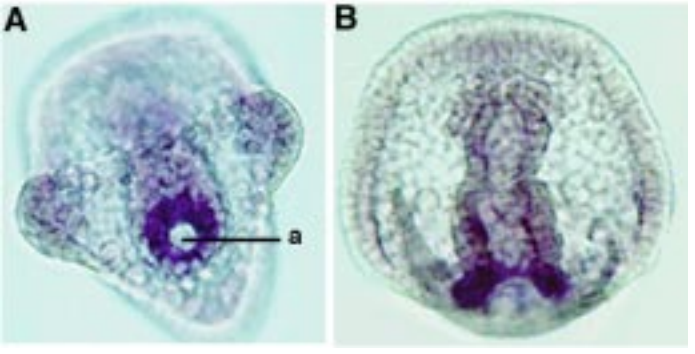

Fig. 2. Pattern of expression of the PlHBox9 gene in late cleavage embryos. *In situ hybridization to* **(A)** *pluteus and* **(B)** *prism stage embryos. Transcripts are localized in few cells located at the base of the archenteron and around the anus (a).*

radial body of the adult (Arenas-Mena *et al*., 1998). It follows that genes belonging to the *Hox* complex are weakly required during early embryogenesis and most of them are strongly expressed once the rudiment has been formed.

Dispersed homeobox genes in Echinoderms

Homeobox genes of the dispersed superclass are expressed in the developing larvae at different stages of development. Studies of spatial expression of homeobox containing genes, suggest a role in the specification of cell fate of the presumptive ectodermal territories at early developmental stages (Di Bernardo *et al.,* 1994, 1995; Gan *et al.,* 1995) or in the control of the terminal state of differentiation of particular cell types and / or the boundary regions in the late embryo (Angerer *et al.,* 1989; Martinez and Davidson, 1997; Dobias *et al.,* 1997; Bellomonte *et al.,* 1998; Di Bernardo *et al.*, 1999).

Additional cues on the role played by those homeobox genes whose spatial pattern in normal development has been extensively investigated, come from mis-expression studies. For instance SpOtx, seems to be involved in the differentiation of the aboral ectoderm territory (Gan *et al.,* 1995; Mao *et al.,* 1996); SpMsx might play a role in vegetal plate specification and primary and secondary mesenchyme patterning (Dobias *et al.,* 1997; Tan *et al.,* 1998); and finally, as it will be described below, *PlOtp* appears to play a key role in skeletal morphogenesis (Di Bernardo *et al.,* 1999).

PlHbox12, *a transiently expressed homeobox gene likely to play a role in conditional cell specification*

Several homeobox genes were cloned from a *P. lividus* genomic library by screening with an oligonucleotide probe corresponding to the helix III of the homeodomain (Di Bernardo *et al.,* 1994). One of those, *PlHbox12,* encodes for a divergent homeodomain weakly related to the paired-like class genes. To our knowledge, *PlHbox12* is the only transcriptional regulator transcribed by the zygotic genome in a highly transient manner. Transcripts are, in fact, detectable from the 4-8 cell stage up to the early blastula stage, reaching the maximum abundance in embryos at the sixth division. This is the time of segregation of the founder cells of the five polyclonal territories. Interestingly, transcription is initiated around the time in which the first founder cell of the aboral ectoderm territory is segregated at the 8 cell stage (Cameron *et al.,* 1990) and strongly depends on signals emanating from the neighbor cells (Di Bernardo *et al.,* 1995). In Fig.1 examples of spatial distribution of *PlHbox12* mRNAs in embryos at 16 cell (A), 32 cell (B) and 64 cell (C) stages are shown. *PlHbox12* is asymmetrically expressed along both embryonic axes. Transcripts are in fact absent from the micromeres at the most vegetal region and preferentially localized toward the animal half of the embryos at all stages. Expression is furthermore restricted on one side of the embryo, presumably corresponding either to the prospective oral or aboral ectoderm territory.

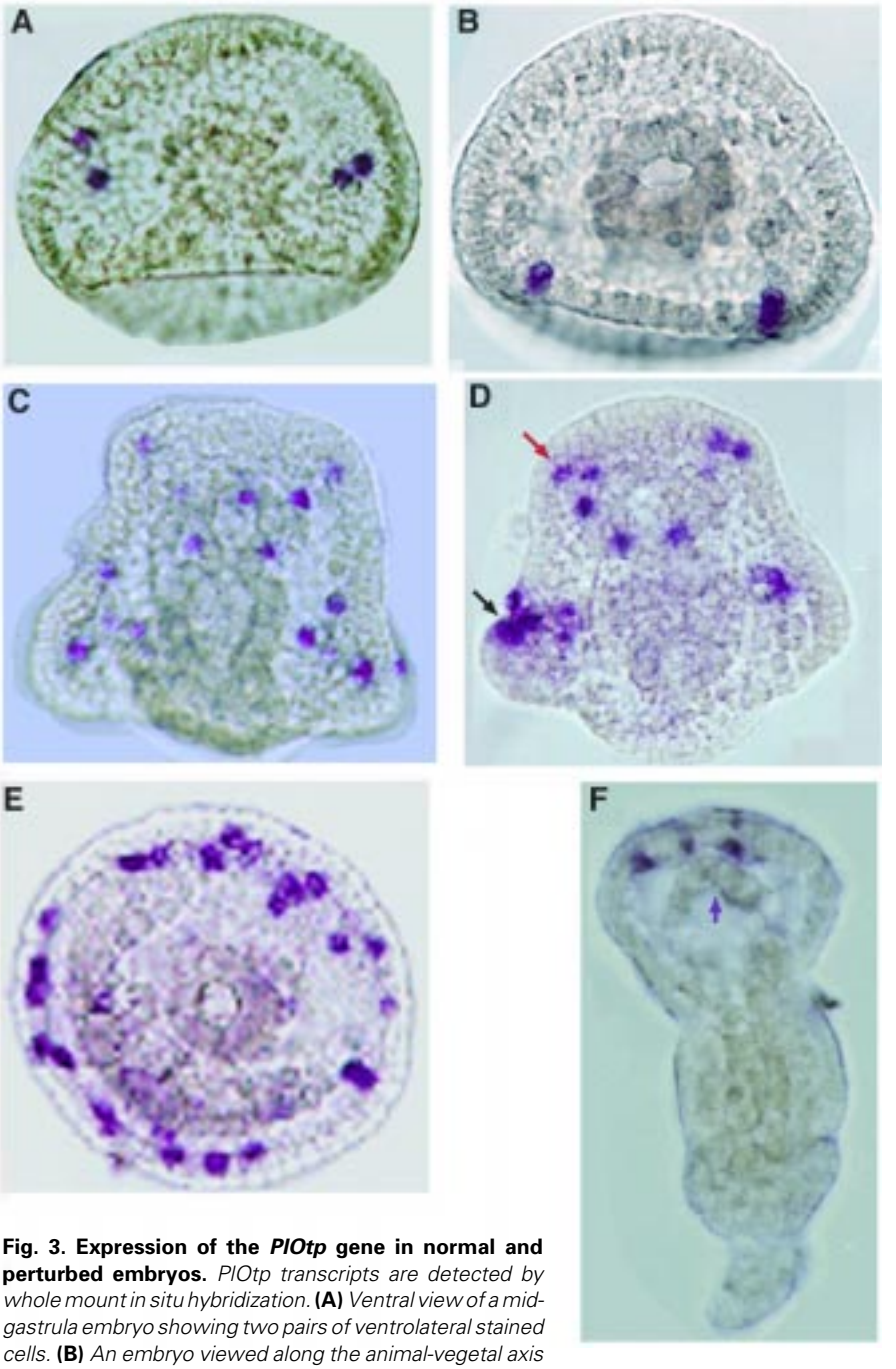

Fig. 3. Expression of the *PlOtp* gene in normal and perturbed embryos. *PlOtp transcripts are detected by whole mount in situ hybridization.* **(A)** *Ventral view of a mid-gastrula embryo showing two pairs of ventrolateral stained cells.* **(B)** *An embryo viewed along the animal-vegetal axis showing that the stained cell are juxtaposed to the PMC clusters.* **(C,D)** *Oral views of prism (C) and early pluteus stage embryos (D) showing expression at the tips of the future anterolateral (black arrow) and postoral (red arrrow) arms and in correspondence to the foregut and the coelomic sacs.* **(E)** *Expression of PlOtp in a nickel -treated embryo. The number of the Otp expressing cells is greatly increased in the radialized embryo.* **(F)** *Otp expression in a lithium-treated embryo is instead limited to a pair of ectodermal cells localised at the most animal pole and overlying the PMC clusters (arrow).*

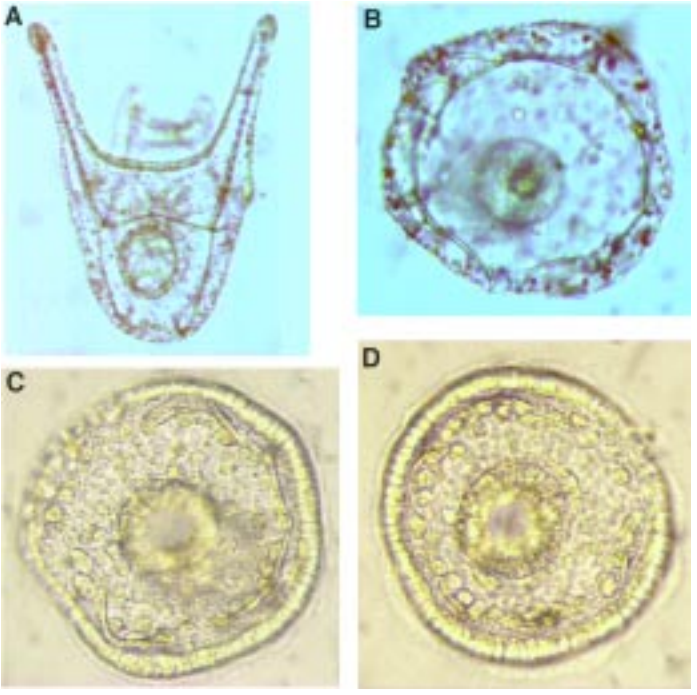

Fig. 4. Phenotypic effects of *Otp* misexpression. (A) *glycerol injected S. granularis embryo at the pluteus stage showing a normal skeleton pattern.* **(B-C)** *radialized S. granularis (B) and P. lividus (C-D) embryos viewed along the animal vegetal axis displaying circular and branched skeleton rods.*

Hbox9, *a boundary specific homeobox gene*

Although *PlHbox12* is, among transcription factors, one of the earliest genes expressed by the sea urchin genome, most of the known homeobox genes to date examined are required late in development. *PlHbox9* has been cloned in our laboratory by the same screening of a genomic library at low stringency described above (Bellomonte *et al.,* 1998). Data bank search has shown that its homeodomain is highly similar to that of the human gene HB9 that is expressed in the lymphoid lineage and in several tissues of the gastrointestinal tract (Harrison *et al.,* 1994). Expression analysis revealed that transcripts appear at the end of gastrulation and increase as development proceeds. *In situ* hybridization on whole mount embryos, shown in Fig.2 A, B, demonstrate a highly restricted expression in embryos at (A) pluteus and (B) prism stages in a limited number of cells at the ectoderm-endoderm boundary, around the anus.

PlOtp, *a homeobox gene that controls skeletogenesis*

PlOtp is an *Orthopedia* related gene (Simeone *et al.*, 1994) that was cloned from a *P. lividus* prism stage cDNA library (Di Bernardo *et al.,* 1999). *PlOtp* is a single copy gene whose expression begins by the mid-gastrula and increases at prism and pluteus developmental stages. The spatial distribution on whole mount embryos is shown in Fig.3. *Otp* expression is characterized by a highly symmetric pattern. Transcripts are strictly confined to two pairs of cells localized in the oral ectoderm of the gastrula embryo (A, B), adjacent to the ventrolateral clusters of the primary mesenchyme cells (PMCs) that form the spicule rudiment (reviewed by Ettensohn *et al.,* 1997). The stained cells are not contiguous one to the other, but instead they are separated by one or two cells that are inhibited from or are not committed to express the gene (A). At later stages their number increases, although being limited to 18-20 cells of the oral surface, always displaying a bilateral symmetric pattern (C, D). Remarkably, the *Otp* expressing cells are located close to the sites of active skeletal growth, i. e., at the tips of anterolateral and post-oral arms (red and black arrows in D). Chemicals that alter polarization along the embryonic axes destroyed this pattern. Thus, $NiCl_2$ treatment known to ventralize the embryo by strongly reducing the aboral ectoderm territory (Hardin *et al.,* 1992), caused an abnormal increase in *Otp* expression in the ectoderm cells radially placed around the archenteron (E). By contrast, expression dramatically diminished in embryos cultured in the presence of LiCl. In these embryos the endoderm domain enlarges at the expense of the ectoderm, which remains confined to a small portion of the embryo at the animal pole. PMCs are shifted along the A-V axis towards the animal pole in proximity of the *Otp* expressing cells (F).

Close inspections of the *in situ* hybridizations on normal and perturbed embryos strongly suggests that *Otp* expression is correlated with active skeleton growth in the oral field. To prove this hypothesis, the *Otp* homeogene was expressed in ectopic positions in both *Spherechinus granularis* and *P. lividus* embryos by microinjecting an excess of *in vitro* transcribed mRNA. Most of the embryos of both sea urchin species resulted completely radialized, with an abnormally developed skeleton (Di Bernardo *et al.,* 1999). In Fig. 4, examples of *Otp* mRNA injected embryos presenting altered phenotypes are shown. The characteristic dorsal-ventral polarity recognisable in normal embryos (A) has been lost and the skeletal elements are radially distributed around the archenteron. At first sight the apparent disruption of the O-A (oral-aboral) axis shown is comparable to that observed in the nickel treated embryo (Fig. 3E). In fact, in either cases multiple *foci* of spicule *primordia* are generated (Hardin *et al.,* 1992; Di Bernardo *et al.,* 1999), most probably as the result of multiple signals emanating from the overlying ectodermal surface.

Our results indicate that in addition to affect skeletal patterning, the *Otp* transcriptional regulator seems involved in determining the fate of ectoderm cells along the O-A axis. In fact , ectopic expression of the *Otp* gene causes the oral ectoderm marker Ecto V to be expressed in an expanded domain, indicating a re-specification of ectoderm cells towards an oral fate (Hardin *et al.,* 1992; Di Bernardo *et al*., 1999). Since similar effects are observed upon nickel treatment, we suggest that *Otp* and nickel, change the commitment of ectodermal cells along the O-A axis through a similar cascade.

Perspective and possible function of homeobox genes in sea urchin development

As demonstrated by experimental evidence, in the sea urchin embryo the first cleavage planes physically create cytoplasmic domains with a different distribution of the maternally inherited molecules. Although no evident hint of differentiation is evident at early stages, these compartments are characterized by differential gene expression and distinct developmental fates of the blastomeres. Good candidates to play a main role in early cell specification are maternal as well as zygotic transcription factors which interact with cis-regulatory systems in the early embryo (reviewed in Davidson *et al.,* 1998). Recruitment of localized

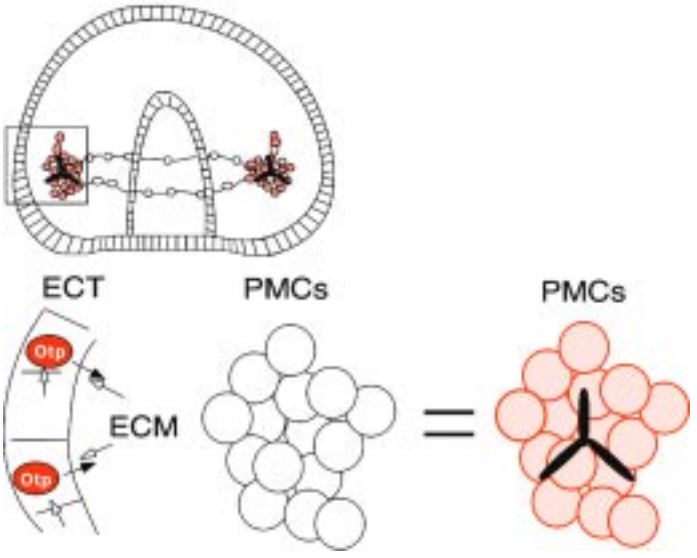

Fig. 5. Model of Otp signalling in the induction of skeletogenesis. *(Top) drawing of a mid-gastrula embryo showing the triradiated spicule primordia at the ventrolateral clusters of PMCs. (Bottom) Enlargement of the ventrolateral region involved in the initiation of spiculogenesis. In two cells of the oral epithelium, marked by a red ellipse, the Otp gene is activated by an external signal emanating from the sorrounding ectodermal cells or from the ECM (white arrows). The Otp homeodomain regulator binds to cis-regulatory sequences of target gene(s), probably encoding for a secreted molecule. The signalling cascade reaches PMCs through the blastocoele and upregulates the synthesis of the SM30 gene, which initiates spiculogenesis.*

transcription factors is certainly needed to activate the homeobox containing gene *PlHbox12*. The early, transient and spatially restricted expression suggest that *PlHbox12* is involved in cell fate specification of the oral or aboral ectodermal territories at the early cleavage stages. Several attempts to prove this hypothesis by microinjection of excess mRNA for ectopic expression have so far failed. Experiments are in progress in our laboratory to construct a *Hbox12-engrailed* repressor chimera that will be (or its mRNA) microinjected. The possible phenotypic effects would then be analyzed. Interestingly, no true *HBox12* related genes have been cloned so far from evolutionary distant developmental systems. Since *HBox12* appears to be expressed only in early development, its function could have been uniquely required in specification processes strictly linked to the invariant cell lineage of the sea urchin embryo. It would be interesting to see if and where *Hbox12* homologues are expressed in other classes of Echinoderms.

PlHbox9 is expressed just after the completion of gastrulation in a narrow stripe of cells that probably constitute and organize a novel spatial boundary which definitely separates the archenteron and the aboral ectoderm (Davidson, 1993). This region, which corresponds to the blastopore, has been suggested to be specified by the time of gastrulation to separate the endoderm and ectoderm fields. Blastopore closure occurs by rearrangement of cells at the blastopore lip (Hardin, 1996). In fact, while ectoderm cells are normally inhibited to form endoderm, gastrula ectoderm cells are competent to be part of the gut, if transplanted in the endoderm domain. Thus the boundary region would prevent the improper recruitment of cells into the gut forming region of the embryo (McClay and Logan, 1996). *PlHbox9,* in sea urchin, could be one of the cues that make this small region different from the surrounding fields, maintaining separated ectoderm and endoderm which must develop their own identity.

The striking expression pattern of PlOtp in the oral ectoderm strongly suggested that expression of the gene is primarily linked to skeletal morphogenesis. Old and new evidence demonstrate that primary mesenchyme cells (PMCs) patterning and skeleton growth are the balanced result of an interplay between PMCs and oral ectoderm (reviewed by Ettensohn *et al.,* 1997). Since PMCs can be cultured *in vitro* and make spicules in the presence of horse serum (Okazaki, 1975), it is very probably that a signaling cascade from the ectoderm is responsible for the expression of skeletogenesis genes, such as SM30 (Guss and Ettensohn, 1997). Interestingly, we have recently obtained compelling evidence strongly suggesting that the expression of the two genes is strictly correlated. In fact, the abundance of the SM30 transcripts dropped to very low levels upon inhibition of *PlOtp* transcription. The effect seems specific since SM50, another PMC marker gene that is not involved in the initiation and growth of spicules (Guss and Ettensohn, 1997), is expressed at levels similar to those of the control (unpublished observations). On the basis of all these data, we propose that *Otp* is upstream the signaling cascade that starts in the oral ectoderm territory. A possible model, which was extended on the basis of a pre-existing one (Ettensohn *et al*., 1997) is shown in Fig.5. At mid-gastrula stage, expression of the *Otp* transcription factor in two pairs of “committed” ectoderm cells of the oral field selectively trans-activates one or more target genes, whose products could be secreted molecules such as growth factor(s). The signaling molecule, after the association to an ECM molecule in the extracellular space, would form an activated complex, able to bind to a receptor on the surface of the PMCs. Finally this signaling cascade up-regulates the synthesis of the SM30 gene, whose expression is known to be strictly correlated with skeletal rod initiation and elongation and influenced by ectodermal cues (Guss and Ettensohn, 1997). Alternatively, secretion of growth factors by the *Otp*-flagged cells could be required for the synthesis or the modification of the ECM molecule, through which the signal is transferred to the target PMCs.

In conclusion, homeobox genes expressed during sea urchin embryogenesis belong primarily to the dispersed superclass. The emerging scenario is that some of these genes have acquired new developmental functions in sea urchin. This is particular evident for *Otp* and *Otd/Otx* related genes that in *Drosophila* and mouse are involved in brain development and patterning of the CNS (Simeone *et al*., 1994; Acampora and Simeone, 1999; Acampora *et al*., 1999). In sea urchin, that has no head, these genes are involved, respectively, in skeletal morphogenesis (Di Bernardo *et al.,* 1999) and aboral ectoderm and vegetal plate specification (reviewed in: Davidson , 1999). It is pertinent to recall here that it has been reported that *distal-less, engrailed and orthodenticle* homeogenes show deep modifications in the symmetry of their expression domains either with respect to other phyla, or among different classes of echinoderms (Lowe and Wray, 1997). Taken together, these results raise the possibility that homeobox genes in echinoderms have acquired new cell type specificity of their expression domains, through the variation of cis regulatory elements.

Acknowledgments

This work was supported by grants of MURST ("Ministero dell' Università e della Ricerca Scientifica e Tecnologica", Cofinanziamenti Programmi di Ricerca Scientifica di Interesse Nazionale, 97 and Ateneo ex 60%). Many thanks are due to Prof. Giudice for reading the manuscript.

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