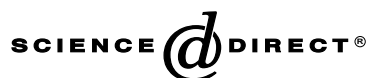


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Activation of *pmar1* controls specification of micromeres in the sea urchin embryo

Paola Oliveri,^a Eric H. Davidson,^a and David R. McClay^{b,*}^a Division of Biology, 156–29, California Institute of Technology, Pasadena, CA 91125, USA^b DCMB Group, Department of Biology, Box 91000, Duke University, Durham, NC 27708, USA

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Abstract

pmar1 is a transcription factor in the paired class homeodomain family that was identified and found to be transcribed in micromeres beginning at the fourth cleavage of sea urchin development [Dev. Biol. 246 (2002), 209]. Based on in situ data, molecular perturbation studies, and QPCR data, the recently published gene regulatory network (GRN) model for endomesoderm specification [Science 295 (2002) 1669; Dev. Biol. 246 (2002), 162] places *pmar1* early in the micromere specification pathway, and upstream of two important micromere induction signals. The goal of this study was to test these three predictions of the network model. A series of embryo chimeras were produced in which *pmar1* activity was perturbed in one cell that was transplanted to control hosts. At the fourth cleavage, micromeres bearing altered *pmar1* activity were combined with a normal micromereless host embryo. If β -catenin signaling is blocked, the micromeres remain unspecified and are unable to signal to the host cells. When such β -catenin-blocked micromeres also express Pmar1, all observed micromere functions are rescued. The rescue includes expression of the primary mesenchyme cell (PMC) differentiation program, expression and execution of the Delta signal to induce secondary mesoderm cell (SMC) specification in macromere progeny, and expression of the early endomesoderm induction signal necessary for full specification of the endoderm. Additionally, Pmar1 expressed mosaically from inserted DNA constructs causes induction of ectopic Endo 16 in adjacent cells, demonstrating further that Pmar1 controls expression of the early endomesoderm induction signal. Based on these experiments, Pmar1 is an important transcription factor necessary for initiating the micromere specification program and for the expression of two inductive signals produced by micromeres. Each of the tests we describe supports the placement and function of Pmar1 in the endomesoderm GRN model.

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Keywords: Gene regulatory network; Sox; Delta; β -Catenin; Paired homeodomain

Introduction

The recently published gene regulatory network (GRN) for endomesoderm specification provides a new focus for our view of early development (Davidson et al., 2002a,b). That view of development is a progression of specification sequences with many inputs, beginning with cytoplasmically localized information, and later with coordinating inputs of short-range signals from other cells. Each level of specification results from a complex integration of transcription factor inputs (Davidson, 2001). The endomesoder-

mal GRN model integrates published fragmentary information regarding endomesoderm specification in sea urchin embryos and is further supported by additional molecules obtained in several differential screens. The many components in the network model are provisionally assigned a position based on a series of expression and perturbation studies. The value of this network is manifold. It provides a template for further studies that will expand the details of specification. It will enable a temporal and quantitative expansion of our understanding, and importantly, it provides a template for many tests of biological functionality. The network currently is provisional since many of the nodes and positions are based on logical interpretation of experimental results. Authentication of the network requires additional research in at least two areas: biological tests of

* Corresponding author. Fax: +1-919-613-8177.

E-mail address: dmcclay@duke.edu (D.R. McClay).

molecular function and *cis* regulatory analyses that will test and challenge predictions of the network. The present set of experiments is among the first of the biological tests that will, along with the *cis* regulatory solutions, vastly reinforce confidence in the authenticity of the network. Other efforts are underway with many genes to establish whether the predicted connections between transcription factors are direct as indicated in the current model, or whether there is a more indirect relationship between genes in question.

The specification of micromeres is a component of the larger endomesoderm GRN. This cell lineage gives rise to the PMCs, the cells responsible for production of the embryonic skeleton. Micromeres have fascinated embryologists for more than 100 years because they are so easily identified at the fourth cleavage as a result of an unequal cell division at the vegetal pole. Bovari (1901a,b), established their lineage and fate. In the 1920s and '30s, Hörstadius carried out numerous blastomere manipulation experiments, including micromere transplantation (reviewed by Hörstadius, 1939). Micromeres transplanted to the animal pole of a host 16-cell-stage embryo induced an ectopic gut that invaginated from the site of transplantation. These results suggested that micromeres somehow induce endoderm and are important for placement of axial properties. In the absence of micromeres, the embryo lacks a gut (after a long delay, the embryo sometimes regulatively compensates and eventually replaces the gut). Ransick and Davidson (1993) later showed that micromeres begin endoderm induction very early, and that the induction of an ectopic gut at the animal pole exhibits expression of the same molecular markers as the endogenous gut. These experiments suggested that micromeres begin their specification sequence immediately after fourth cleavage and that micromeres are an important source of an early inductive signal (ES) for the endomesoderm lineage.

Embryological experiments demonstrated that micromeres themselves require no further input from the rest of the embryo beyond fourth cleavage since micromeres, isolated at the 16-cell stage, differentiate as PMCs and make skeletal spicules in culture (Okazaki, 1975). These cells require horse serum, so a caveat of the above experiment is the potential requirement for growth factors or other factors present in the horse serum. Nonetheless, numerous experiments have shown that micromeres isolated at fourth cleavage and transplanted to host embryos give rise exclusively to skeletogenic progeny, no matter where they are placed, so by fourth cleavage, in the context of the living embryo, they display autonomous specification. At the 16-cell stage, the nuclei of micromeres become positive for β -catenin, a transcriptional cofactor in the wnt signaling pathway (Logan et al., 1999; Peifer et al., 1991), and β -catenin is necessary for micromere specification (Emily-Fenouil et al., 1998; Logan et al., 1999; Wikramanayake et al., 1998). Though β -catenin is the earliest known input in micromere specification, other components of micromeres have been found to participate in steps that lead to PMCs. Several transcription

factors are expressed exclusively in the micromeres, and perturbation of these has substantial effects on PMCs (Kurokawa, 1999; Davidson et al., 2002b; Amore et al., 2002). The endomesoderm GRN models a hierarchical pattern of gene activation and repression based on perturbation of one and observing the effect on expression of other transcription factors known to be in the micromeres. Based on logic, these studies reveal that maternal nuclear β -catenin and Otx activate transcription of *pmar1* at fourth cleavage, and Pmar1 then initiates the micromere specification sequence. *pmar1* is proposed to be a direct target for the β -catenin, and is further proposed to be upstream of the early endoderm induction signal (ES), the expression of Delta, and the transcriptional apparatus that specifies the differentiated PMCs. Several substantial pieces of information lead to this model. First, Pmar1 is expressed specifically in micromeres beginning at the fourth cleavage, so it is expressed in the right place at the right time. Second, experimental activation of *pmar1* in other parts of the embryo causes an ectopic specification of mesenchyme-like cells. Third, upregulation of *pmar1* leads to upregulation of other micromere lineage transcription factors, but the reciprocal activation is not true (Oliveri et al., 2002).

A later functional property of micromeres is the expression of the Delta ligand to activate Notch in veg 2 cells, a necessary step in the induction of SMCs (Sherwood and McClay, 1999). Delta is released between the 8th and 10th cleavage and is expressed by micromeres at the right time for this signal to activate Notch on the adjacent veg 2 cells (Sweet et al., 2002). Thus, shortly after the micromeres initiate their own specification, they begin to signal to other lineages in the embryo. Ransick and Davidson (1993) showed that a different micromere signal induces endoderm beginning between the 4th and 6th cleavage.

Given this rich background, there are two sources of information necessary to validate the GRN model for the micromeres and extend it. First, since many of the perturbation studies utilize injections that alter gene regulation in the entire embryo, biological studies are necessary to authenticate the function of the GRN in the specific cells where specification events are thought to occur. Second, *cis* regulatory analysis of *pmar1* and other genes in the micromere GRN will strengthen confidence in the proposed transcriptional activation sequence. The purpose of this paper is to test experimentally the micromere portions of the GRN, especially as they relate to *pmar1* function, using blastomere transplantation approaches. Experiments show that all three major functions of the micromere progeny are dependent on *pmar1* function. The ES and the Delta signal are downstream and essentially independent of the regulatory apparatus controlling PMC differentiation. Thus, we confirm that *pmar1* activation is one of the most crucial steps in the activation and specification of the micromere lineage.

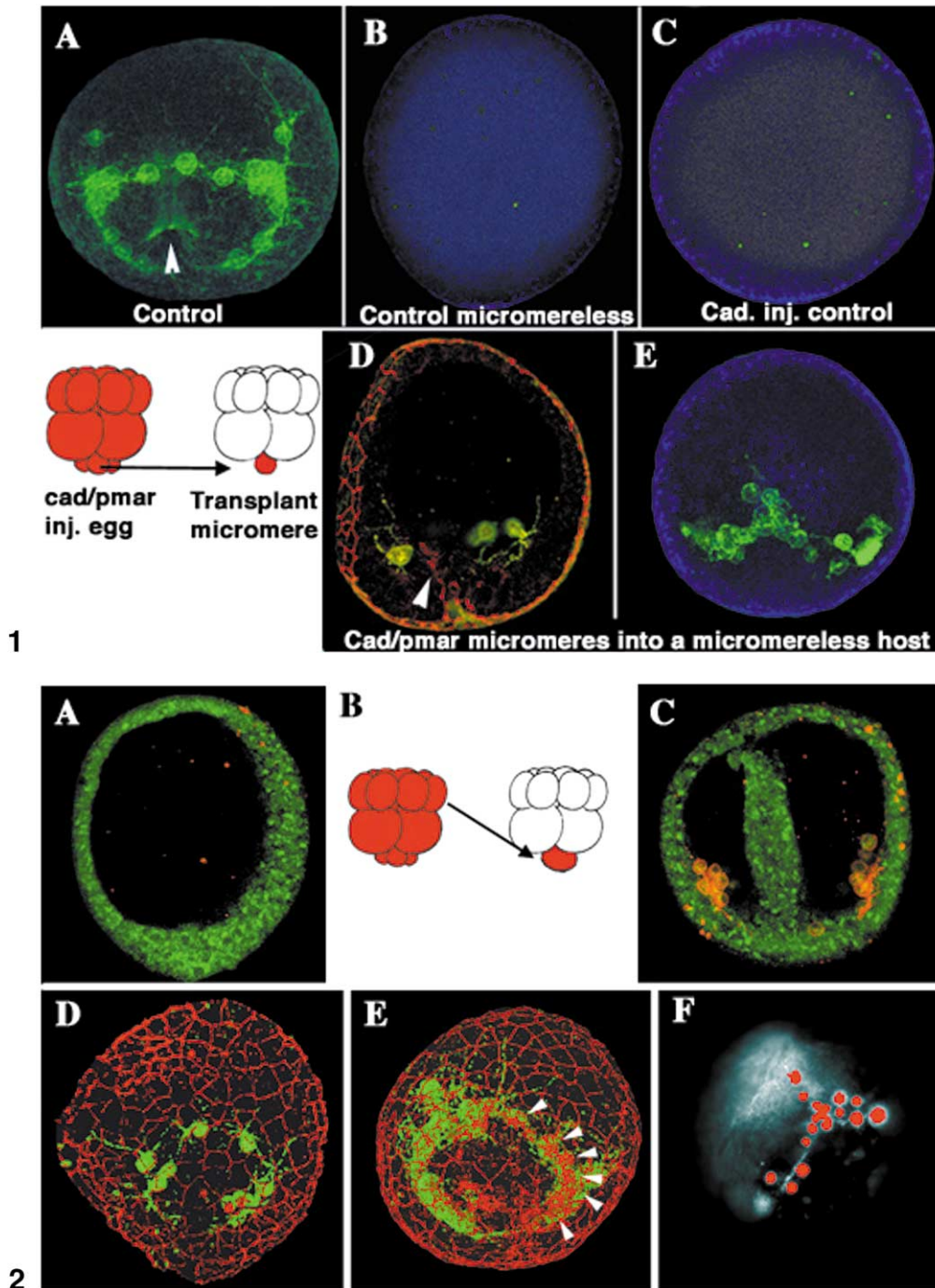


Fig. 1. *Pmar1* rescues embryos in which β -catenin nuclearization is prevented. All embryos at 36 h. (A) Control embryo. PMCs are stained with a PMC marker, and in the center of the PMC ring, the blastopore of the archenteron is visible (arrowhead). (B) An embryo as in (A) but with micromeres removed at the 16-cell stage. The embryo has no PMCs, and no archenteron (16/16 cases). (C) Micromeres from embryos injected with Δ -cadherin were returned to a micromereless embryo as in the inserted diagram, grown to 36 h, then stained with the PMC marker. There were no PMCs and no archenterons (35/36 cases). Control micromeres added to micromereless embryos resembled control embryos with PMC rings and archenterons (17/17 cases; data not shown). (D, E) Micromeres from embryos injected with both Δ -cadherin RNA and *pmar1* RNA were transplanted to the vegetal pole of micromereless control embryos. The embryos had PMC rings and archenterons (arrowhead in D) (29/30 cases). Green stain is a PMC marker; red stain (in D) or blue stain (in A–C and E) is directed against β -catenin in adherens junctions. In all experimental cases, one micromere was transplanted to the vegetal pole of a micromereless host.

Fig. 2. *Pmar1* expression is sufficient to specify any blastomere as a skeletogenic mesenchyme cell precursor. (A) Host micromereless embryos that do not receive micromeres at the vegetal plate have no PMCs and fail to gastrulate by 38 h (18/18 cases). (B) The experiment. Eggs were injected with Δ -cadherin RNA, *pmar1* RNA, and rhodamine dextran. At the 16-cell stage a single, double-injected mesomere (red cell in diagram) was transferred to the vegetal pole of a micromereless control embryo. (C) The *Pmar1*-expressing mesomeres ingress as PMCs, stain with a PMC marker (red), and the archenteron is normally completed by 38 h (17/18 cases). (D) Ingressed mesomeres (stained green with a PMC marker) form a normal-looking PMC ring. The red stain is β -catenin in adherens junctions. (E) When transplanted to the vegetal plate of embryos with endogenous micromeres, the double-injected mesomeres still make PMCs, (arrowheads pointing to red-stained mesomere-neo-PMC cells) and join into the vegetal plate ring along with the endogenous PMCs. (F) The skeleton produced by a *Pmar1*-expressing mesomere transformed into a PMC is entirely of mesomere origin (rhodamine-labeled mesomere cells are pseudocolored in the host embryo, and an additional spicule is out of focus in the background).

Materials and methods

Micromanipulation and imaging

Eggs of *Strongylocentrotus purpuratus* were shed in SW following injection of animals with 0.5 M KCl. Eggs were washed in SW and fertilized with dilute sperm in SW containing para-aminobenzoic acid (15 mg/100 ml SW). Cultures were incubated at 16°C. Just prior to surgery, the embryos were passed through 102-micron Nitex mesh four times to remove fertilization envelopes. Alternatively, if embryos were previously injected with RNA, they were pipeted into a narrow bore pipet that manually shears off the fertilization envelope without damaging the embryo. Surgeries were adapted from McClay et al. (2000). Briefly, to transplant cells from *S. purpuratus*, it was necessary to pretreat the embryos by incubation for 30 s in HEM (McClay, 1986), containing 2 mg/ml BSA (HEM/BSA). The BSA eliminates problems with the very sticky hyaline layer surrounding the *S. purpuratus* embryo, and the HEM softens the hyaline layer so that cells can be transplanted.

Embryos to be manipulated were transferred from the HEM/BSA to modified Kiehart chambers (Kiehart, 1982). The chambers were modified so that two glass needles could be inserted into the chamber at 90° to each other. One of the glass needles served as a suction pipet, and the other needle served as a holding and manipulating pipet. These were driven by two joystick micromanipulators. The suction applied was by mouth. For cell transplantations, donor cells were pulled into the suction pipet one at a time. The bore of the pipet was preadapted to a slightly smaller I.D. than the diameter of the cell to be transplanted so that inside the pipet the cell took on a sausage shape. The cell was transferred to a host embryo and inserted into position. The sausage shape helped with the insertion between cells in that the donor cell overlapped initially both to the inside and the outside of the host cell layer. Within a minute, the donor cell rounded up and was retained in position by the host. Embryos were then transferred back to SW. For all operations, the embryos were in HEM for less than 5 min each, and in sham control cell to control embryo operations, development was indistinguishable from unoperated control embryos both in timing of morphogenetic events and in final phenotype.

Several operations were performed. Many of the experiments required that host embryos were first made micromereless. Micromeres were removed early in the 16-cell stage and the embryos were inspected to assure that all 4 micromeres were removed. Donor cells were inserted in the cleft between the 4 macromeres. When these embryos were inspected at the 32-cell stage, the transplanted donor cells were in the same position as control micromeres relative to macromeres. Because the insertion cleft is small, generally only 1 or 2 of the 4 micromeres were transplanted. Nevertheless, previous work demonstrated that 1 micromere conveys an inductive signal that is virtually indistinguishable

compared with the normal 4 micromeres (with only a reduction in the number of pigment cells indicating a reduced level of induction) (McClay et al., 2000).

When mesomeres were transplanted to the vegetal or animal poles, they were taken from the donor embryo at the 32-cell stage and 1 mesomere daughter was transplanted into the same position as micromeres. When mesomeres (or micromeres) were transplanted to the animal pole, they were inserted between the 4 blastomeres closest to the animal pole, again inserting the sausage-shaped cell into the cleft. The transplanted donor cells were pre-labeled with rhodamine dextran (10,000 mw; 1:5 dilution of a 50-mg/ml stock into the injection medium). We avoided the lysinated form of rhodamine dextran, which prevented the dye from being fixed with the tissue; in preliminary experiments, the high lysine content of the fixable dye tended to reduce the effectiveness of injected molecules.

Prior to fixation, the embryos were examined by fluorescent microscopy and scored for phenotype. They were then fixed for 20 min with 2% paraformaldehyde in SW, post-fixed 5 min in MeOH (on ice), and washed into SW containing 4% normal goat serum (SW-NGS). Throughout antibody-staining reactions, the embryos remained in SW-NGS and all antibodies were diluted in this solution. Embryos were stained with antibodies to PMCs [1D5, a monoclonal recognizing MSP-130 (Wessel and McClay, 1985)], β -catenin (Miller and McClay, 1997a), cadherin (Miller and McClay, 1997b), SoxB1 (Kenny et al., 1999), pigment cells (Gibson and Burke, 1985), and Notch (Sherwood and McClay, 1997). Embryos were examined by confocal microscopy using either a Zeiss 410 or 510 scanning confocal microscope.

DNA and RNA constructs and injection of into eggs

A Hatching Enzyme promoter-*pmar1* DNA construct was engineered by replacing the GFP coding region already present in a HE-GFP clone described by Bogarad et al. (1998). The GFP was removed by using the *NheI* and *XhoI* unique sites present in the construct, and the coding region of *pmar1*, obtained by digestion with *SpeI* and *XhoI*, was inserted (Oliveri et al 2002). The subclone was checked by restriction digestion analysis of known sites and by sequencing. The DNA clone obtained was linearized by using *KpnI* and injected as described by McMahan et al. (1985), with the exception of the glycerol in the injection solution.

RNAs for injection were synthesized as described by Oliveri et al. (2002). For micromere rescue and blastomere micromanipulation experiments, RNA was injected along with rhodamine dextran (10 pg/pl; Sigma). The injection concentration of the probes was: Δ -*cadherin* RNA (60 ng/ μ l), *pmar1* RNA (5.6 ng/ μ l) for both the single injection and the coinjection experiments. The injection volume for each egg was approximately 2 pl. In experiments to determine the minimum amount of *pmar1* sufficient to rescue the

Δ -cadherin phenotype, Δ -cadherin RNA at 60 ng/ μ l was coinjected with *pmar1* RNA at 5.6, 2.8, 1.1, and 0.56 ng/ μ l.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described by Ransick and Davidson (1995), with the following modifications. The *endo16* probe was transcribed in presence of 10 \times DIG RNA labeling mix (Roche) and the GFP probe in presence of 10 \times Fluorescein RNA labeling mix (Roche). The two probes were hybridized to fixed embryos at the same time under the conditions described by Oliveri et al. (2002). The detection of the two different probes was obtained following the conditions described by Hauptmann (2001) and using the NBT/BCIP ready-to-use tablet (Roche) for the DIG-labeled probe and the Fast Red (Roche) for the Fluorescein-labeled probe.

Results

Pmar1 expression rescues cadherin-inhibited specification of micromeres

β -Catenin enters the nuclei of micromeres shortly after their birth at the fourth cleavage (Logan et al., 1999). Experimentally, the nuclear entry of β -catenin and its subsequent association with TCF-Lef (Vonica et al., 2000) is required for micromere specification. If nuclear entry of β -catenin is blocked by expression of the cytoplasmic tail of cadherin (Wikramanayake et al., 1998), or by blocking GSK-3 (Emily-Fenouil et al., 1998), micromeres fail to be specified. Oliveri et al. (2002) found that one of the first markers to be expressed in micromeres following nuclear entry of β -catenin is *pmar1*, a transcription factor of the paired homeodomain family. *pmar1* expression is greatly diminished if β -catenin fails to enter the micromere nuclei. Manipulation of *pmar1* in whole embryos demonstrated that it acts as a repressor. The most direct evidence for this is that introduction of mRNA encoding an Engrailed (*en*) repressor domain with *pmar1* homeodomain fusion forces the Pmar1-*en* fusion to act as an obligate repressor for target genes. This construct produces identical effects to introduction of mRNA encoding the natural Pmar1 protein. Both cause all downstream micromere genes to be globally repressed. Therefore, the micromere-specific expression of the *pmar1* gene normally has the effect of repressing a gene encoding a repressor of these downstream genes. It thereby allows them to become active exclusively in micromeres and their descendants. In addition, the Pmar1 protein includes a sequence motif that shows similarities to several well-known transcriptional repressors (Oliveri et al., 2002).

To ask how *pmar1* works in the micromeres, we began a series of studies in which expression of *pmar1* was modified only in the micromeres. Three functions were examined: (1) expression of the ES (Ransick and David-

son, 1995), a signal necessary for correct specification of the endomesoderm and for on-time invagination of the archenteron, (2) expression of the Delta signal, necessary to activate Notch in the veg 2 cells to specify SMCs (Sherwood and McClay, 1999; Sweet et al., 2002); and (3) differentiation of micromere progeny into skeletogenic mesenchyme cells. Earlier transplantation studies showed that, if normal micromeres are transplanted from a donor 16-cell-stage embryo to a host micromereless embryo, the micromeres behave just as they do in an unmanipulated embryo (McClay et al., 2000). If a micromereless host received transplanted micromeres, the micromeres ingress as PMCs, express surface molecular markers, and produce a skeleton. The embryo gastrulates at the correct time (32–36 h for *S. purpuratus* at 16°C), SMCs are specified normally, based on the appearance of pigment cells by 48 h, then coelomic pouches, a muscular pharynx, and blastocoelar cells in pluteus larvae at 72 h. If, in the same operation, micromeres are not replaced, as in Fig. 1B, most embryos fail to gastrulate. In a large number of trials, many fewer than 20% of the micromereless embryos eventually gastrulate. Those few embryos that eventually recover and gastrulate become albino pluteus larvae, their archenteron invagination is delayed to beginning at 55–72 h, and PMCs plus nonpigmented SMCs become replaced only in those embryos that eventually gastrulate. Thus, gastrulation and specification of SMCs are severely compromised by the absence of micromeres. In embryo recombinations, when β -catenin-induced input was eliminated from micromeres by transplantation of Δ -cadherin-expressing micromeres to replace normal micromeres on uninjected host embryos, the embryos developed as if they were micromereless (Fig. 1C). The chimeras lacked PMCs, invagination of the archenteron was greatly delayed if it occurred at all, and all such embryos were albino, presumably as a consequence of a failure of the micromeres to express Delta.

When *pmar1* RNA was injected along with the same β -catenin-inhibiting concentration of Δ -cadherin, and micromeres transplanted at the 16-cell stage, normal-appearing development returned (Fig. 1D and E). The double-injected micromeres became PMCs, a skeleton formed, and archenterons invaginated at the correct time (32–36 h), in almost all of the transplant combinations (greater than 95% of 136 transplant combinations in 19 separate experiments) (Fig. 1D and E). When embryos were cultured longer, they displayed the full range of SMCs, including pigment cells (see Fig. 3 below), a muscular pharynx, coelomic pouches, and blastocoelar cells. Thus, expression of *pmar1* rescued all the known functions of micromeres, including production of both induction signals and the production of a normal skeleton. Whole embryos that expressed *pmar1* alone or Δ -cadherin plus *pmar1* formed a mass of mesenchymal cells.

The Pmar1 rescue of micromeres is effected by fewer copies of RNA than normally are synthesized by micromeres

A general concern of the RNA injection experiments, like the rescue experiment described above, is that the phenotype observed could be caused by a vast overexpression of *pmar1* that forces an artifactual conversion of cells to the mesenchyme phenotype. Although the Δ -*cadherin*:*pmar1* ratio of injected mRNA was 10:1, the described rescue of the β -catenin-devoid phenotype was obtained with the injection of roughly 20- to 40-fold excess amount of *pmar1* mRNA into whole eggs, relative to calculated amounts of endogenous *pmar1* RNA in micromeres (see Materials and methods for injection conditions). If one conservatively considers no degradation of the injected RNA and assumes equal partitioning of the injected RNA throughout the cytoplasm, the micromeres at the moment they are born (4th division) will inherit just 1.6- to 3.2-fold excess *pmar1* mRNA relative to untreated embryos [the micromeres segregate 8% of egg cytoplasm (Davidson, 1988)]. Thus, the observed rescue is unlikely to result from vast overexpression of *pmar1*.

To experimentally establish the minimum amount of *pmar1* necessary to rescue micromere specification, we analyzed effects of a dilution series of RNA injections. One might predict that the dominant repressor function of Pmar1, to extinguish a ubiquitous repressor and thereby activate the micromere-PMC cell program, can be reached with a relatively low number of transcripts. To test that hypothesis, we determined the smallest amount of *pmar1* necessary to overcome the Δ -*cadherin*-altered phenotype. *pmar1* RNA at increasing dilutions was injected into eggs along with the minimum number of molecules of Δ -*cadherin* RNA known to eliminate β -catenin signaling (Δ -*cadherin*:*pmar1* ratio of transcripts 20:1, 40:1, and 100:1). Embryos were scored at 24 h of development for the resulting phenotype. Using this approach, we find the minimum number of *pmar1* copies/cell necessary to rescue the micromere specification, as well as transfect the other blastomeres, is 0.4- to 0.8-fold the normal amount of *pmar1* RNA present in micromeres at 5th cleavage. This corresponds to about 66–130 copies per micromere relative to 163 copies/cell maximally present in normal embryos between the 4th and 8th cleavage (Oliveri et al., 2002). Thus, *pmar1* rescues micromere specification even when present at a fraction of levels expressed in control embryos.

Ectopic Pmar1 expression specifies the micromere program in all cells of the embryo

In micromeres, the endomesoderm GRN predicts Pmar1 to repress a ubiquitous repressor expressed everywhere in the early embryo. If this ubiquitous repressor normally blocks micromere specification elsewhere in the embryo as well, the model predicts that ectopic expression of *pmar1*

should force nonmicromeres into the micromere specification pathway. The mesenchymal phenotype of *pmar1* mRNA-injected whole embryos tends to support this hypothesis. But, are those Pmar1-expressing cells converted to functional micromeres? To address this question, *pmar1* RNA was either injected with Δ -*cadherin* RNA or alone (with an identical phenotypic outcome). At the 16-cell stage, a single *mesomere* (or *macromere* in other cases) was removed from the animal pole of injected embryos and transplanted to the vegetal pole in place of four removed micromeres of an uninjected control embryo. Mesomeres normally produce only oral and aboral ectoderm. Fig. 2 shows the remarkable result that the progeny of the transplanted Pmar1-expressing mesomeres behave in almost every way like micromeres. The Neo-PMCs of the chimera ingress, the archenteron invaginates at the correct time demonstrating that the transplanted mesomere conveys the ES (Fig. 2C). The transformed mesomeres make a normal-appearing PMC ring (Fig. 2D and E), and they make a skeleton (Fig. 2F) (18/18 cases). When examined at 48 h., embryos with transformed mesomeres are pigmented (Fig. 3C), suggesting that the Delta ligand was produced correctly (4/6 cases), while control micromereless embryos (18/18), micromereless embryos that received uninjected mesomeres (9/9), or micromereless embryos that received mesomeres with Δ -*cadherin* only (6/6), failed to gastrulate, failed to make PMCs, and failed to make pigment cells (data not shown). Pluteus larvae with Pmar1-transformed mesomeres were observed to have a muscular pharynx, coelomic pouches, and blastocoelar cells, indicating that the full range of SMCs was specified (data not shown). The only abnormal aspect of the mesomere-PMC conversion observed was the somewhat abnormal pattern of the skeleton. With this exception, early ectopic *pmar1* expression is able to convert a mesomere from its normal ectodermal fate into cells of the micromere-PMC lineage, and the conversion is almost complete. A similar outcome is observed if *pmar1*-injected macromeres are transplanted to the vegetal plate (data not shown). We conclude that expression of *pmar1* is sufficient to launch the micromere specification program. Since any cell expressing Pmar1 is specified as a micromere/PMC, we suggest that no other maternal components are necessary in micromeres other than those that activate *pmar1*.

Pmar1 expression in micromeres is upstream of the two signals that induce endoderm and secondary mesoderm

The SMC induction signal, Delta, is expressed by micromeres between the 8th and 10th cleavage (McClay et al., 2000; Sherwood and McClay, 1999; Sweet et al., 1999 2002). Previous work showed that ectopic *pmar1* expression activates ectopic *delta* RNA expression (Oliveri et al., 2002). To confirm that Pmar1 is upstream of Delta, and importantly, that the Delta produced in response to *pmar1* activation has SMC inductive activity, we manipulated *pmar1* in micromeres and mesomeres as above and then

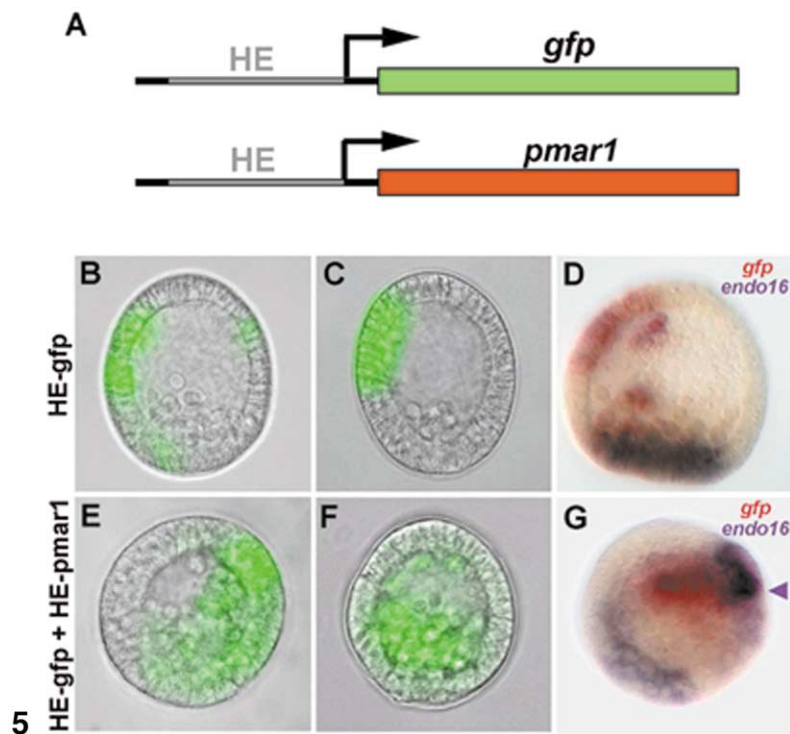
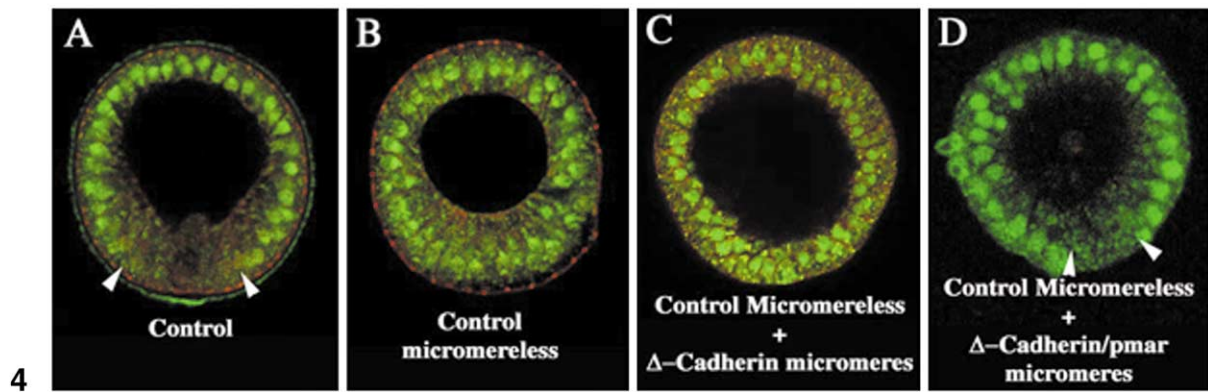
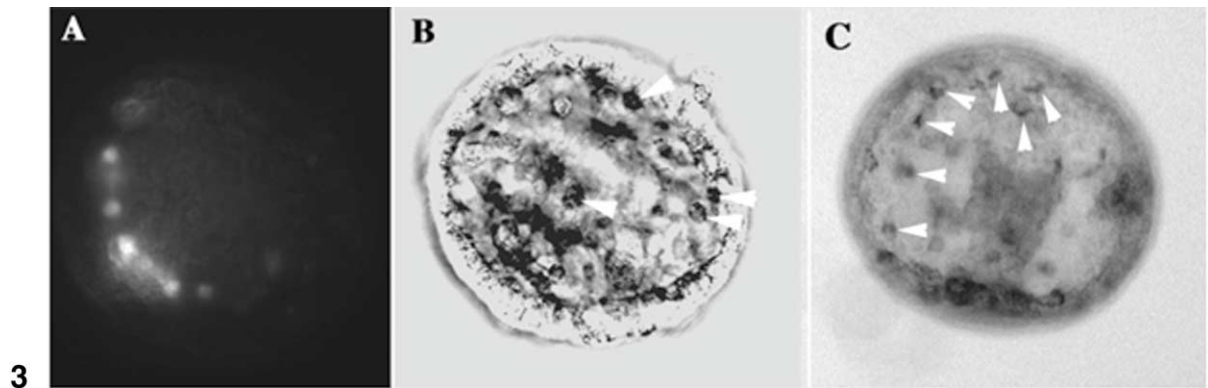


Fig. 3. *Pmar1* expression is necessary for production of the SMC induction signal. (A, B) *cad/pmar1* double-injected micromeres were transplanted to the vegetal pole of control micromereless host embryos. (A) At 48 h, the *cad/pmar1*-injected cells were PMCs (Portion of ring of fluorescent cells in A). (B) At the same plane of focus, that embryo also had nonfluorescent (i.e., induced) pigment cells (arrowheads) (5/6 cases examined). (A) Epifluorescent image of the same embryo showed in (B) (bright field). (C) *pmar1*-expressing mesomeres transplanted to the vegetal pole of micromereless embryos also induce the host to produce pigmented cells (arrowheads) (4/6 cases examined). Control micromereless embryos were albino (6/6 cases examined in this experiment; data not shown).

Fig. 4. *Pmar1* is upstream of the early endoderm induction signal. An assay for a consequence of the ES signal examines nuclear SoxB1 immunocytochemically (green nuclei). In each case, PMCs were removed as micromeres or ingressed, leaving only endomesoderm at the vegetal plate (with the exception of C).

examined the ability of those micromeres, when transplanted to the vegetal plates of unmanipulated micromereless embryos, to induce pigment cells. Fig. 3 shows that when Pmar1 is present in the transplanted micromeres, a pigmented embryo results by 48 h. (23/30 cases). Embryos are albino in the absence of *pmar1*-expressing micromeres (6/6 analyzed cases). Manipulation of *pmar1* in micromeres has the predictable effect on that induction sequence (no *pmar1* expression, no induction). Embryos with *pmar1*-expressing mesomeres also produce pigment cells by 48 h (Fig. 3C). If grown to the pluteus stage, embryos with transplanted Pmar1-expressing micromeres or mesomeres also have a muscular pharynx, coelomic pouches, and blastocoelar cells (data not shown). We conclude that *pmar1* expression is sufficient for the production of the Delta induction signal, a signal that is necessary for SMC specification.

The early endomesoderm induction signal (ES) is first detected between the fourth and sixth cleavage (Ransick and Davidson, 1995), i.e., if micromeres are present during that time *endo 16* message is later detected normally in the vegetal plate. If micromeres are removed, *endo 16* expression is reduced progressively depending on whether micromeres are removed at the 6th, 5th, or 4th cleavage. The provisional endomesoderm GRN proposes *pmar1* expression to be required for rapid activation of the ES in the micromeres (Davidson et al., 2002a,b). The next tests examined this prediction that place *pmar1* immediately upstream of the production of the ES.

To examine the ES, two assays were developed that require this signal. The first assay measured disappearance of nuclear SoxB1 (Fig. 4). SoxB1 is a transcription factor present maternally in all cells (Kenny et al., 1999). Early in development, maternal SoxB1 protein concentration is greatly reduced in micromere nuclei, and after hatching is cleared from the nuclei of veg 2 cells and ultimately to the entire endomesoderm region. Fig. 4 shows that SoxB1 clearance requires the presence of micromeres and that requirement fits parameters of the ES. An assay was designed, using immunochemical disappearance of nuclear SoxB1 from the veg 2 cells as a measure, to examine the

proximal consequences of the ES (Fig. 4). The ES also is a prerequisite for on-time gastrulation and for later expression of *endo 16* in the vegetal territory, providing two additional assays to assess ES activity (Ransick and Davidson, 1995) (Fig. 5). We used both of these assays to ask if *pmar1* expression in cells is necessary for the ES response in neighboring cells.

If micromeres are absent, there is no SoxB1 clearance from the vegetal plate (Fig. 4B) at 20 h (early mesenchyme blastula stage), and the archenteron fails to invaginate until at least 24–36 h beyond normal invagination time in a small percentage of embryos. Control embryos with micromeres eliminate SoxB1 from a large region of the vegetal plate that is about 13 cells wide, as counted from confocal sections (Fig. 4A), and gastrulate beginning at 30–32 h. If Δ -*cadherin*-expressing micromeres are transplanted to micromereless control embryos, SoxB1 clearance from endomesoderm fails to occur (Fig. 4C) and the archenteron fails to invaginate. If the transplanted micromere expresses a rescuing level of Pmar1, SoxB1 is cleared from the veg 2 cells, indicating that the ES is produced and functional (Fig. 4D), and the archenteron begins invagination at 30–32 h.

Fig. 5 demonstrates that *pmar1* is upstream of the early signal using a different experimental approach. The entire coding region of *pmar1* was placed under the control of the hatching enzyme (*HE*) *cis* regulatory element. The *HE* promoter is a well-characterized regulatory region that allows expression of a downstream gene everywhere in the embryo except in the most vegetal territories (Wei et al., 1997). Groups of 200–300 eggs were injected with constructs: hatching enzyme promoter-driven *pmar1* (*HE-pmar1*) and/or the hatching enzyme promoter-driven *gfp* (*HE-gfp*) (Bogarad et al., 1998). Double injected embryos concatenate the two DNA constructs and express them mosaically, in the same cell(s) (Arnone et al., 1997). Thus, if double injected, a *gfp*-expressing clone of cells will also express *pmar1*. The easily detected GFP therefore is used as an ectopic *pmar1* expression marker. As shown already by the whole embryo mRNA injections, all cells of the embryo have the capability of acquiring a micromere fate if they express *pmar1* (Oliveri et al., 2002). Therefore, ectopic cell

(A) Normal SoxB1 clearance from the endomesoderm cells of the vegetal plate by 20 h (316/316 controls examined in 19 experiments). (B) If micromeres are removed during 4th cleavage, SoxB1 fails to clear from the endomesodermal vegetal plate (34/36 cases examined). (C) If micromeres lacking β -catenin input are present at the vegetal plate, those micromeres fail to convey the ES, and no SoxB1 is cleared from vegetal plate nuclei (3/3 cases examined) (D) Pmar1 expression by transplanted micromeres rescues the ES as seen by the vegetal clearance of SoxB1 from endomesoderm. The Pmar1-expressing PMCs ingressed (as they did in controls) (11/12 cases examined). All embryos shown were fixed at 20 h of development. Micromeres were labeled with rhodamine dextran to track location of micromere progeny. Prior to fixation, the location of PMCs was noted; then, with fixation, the red dye was lost from the cells. Fig. 5. Mosaic expression of *pmar1* in ectopic positions leads to endoderm induction in adjacent cells. (A) Schematic map of the two constructs used to induce mosaic ectopic *pmar1* and *gfp* expression via DNA injection. Both the *pmar1* coding sequence (red) and the *gfp* sequence (green) were placed under the control of the hatching enzyme promoter (HE). The hatching enzyme regulatory sequence normally drives expression ubiquitously, except in micromeres. (B, C) Embryos injected only with *HE-gfp* construct. As expected, GFP was expressed mosaically in patches of cells, except by PMCs as indicated by the green false color, in 95% of the cases. (E, F) Eggs received *HE-gfp* and *HE-pmar1* DNA. Again, GFP was expressed in a mosaic pattern (green cell clones), but in these embryos, GFP was expressed abundantly in PMCs, indicating that, wherever *pmar1* is expressed, the cells were transformed into PMCs. Note that the embryos show extra PMCs and a reduced blastocoel. In (D), eggs were injected with the *HE-gfp* construct and were examined by double in situ analysis at mesenchyme blastula stage with probes to *gfp* (brown-red) and an endoderm marker, *endo16* (blue-purple). The *gfp* is expressed only in non-PMC tissues, and *endo16*, as expected, is expressed in the vegetal plate only. By contrast, in (G), eggs that had been double injected with the *gfp* and *pmar1* constructs express *endo16* (blue-purple) in an extra patch of cells (purple arrowhead) adjacent to *gfp* (and *pmar1*)-expressing cells ingressed in the blastocoel.

clones expressing *pmar1* should exhibit micromere functions, including release of induction signals. Fig. 5B and C shows that, when the GFP reporter is expressed alone, it is expressed mosaically in nonmicromeric regions in all of the analyzed cases, as expected. The HE promoter drives the expression of the GFP either in single clones (Fig. 5C) or in multiple different clones (Fig. 5B) in 85–99% of the injected embryos, depending on the embryo batch. By contrast, at 24 h of development, GFP-expressing cells in the double injected embryos ingress into the blastocoel reflecting the behavior of PMCs (Fig. 5E and F). These embryos, in 86% of the cases, show reduced blastocoelar cavities with extra mesenchyme cells in them. Thus, the HE promoter activates *pmar1* expression ectopically. This activates the micromere-PMC regulatory program, and as a consequence, GFP now is expressed in mesenchyme cells.

The next test asked if those double-injected, transformed cells convey the early induction signal. Ectopic expression of *pmar1* not only should convert cells into the micromere lineage as described above, but also should induce adjacent cells to be specified as endomesoderm. This prediction was tested by double in situ detection of *endo16*, an early endomesoderm marker, and *gfp* at 24 h of development after coinjection of *HE-gfp* and *HE-pmar1* (on 66 embryos) or *HE-gfp* alone as control (on 58 embryos). In Fig. 5G, the *gfp/pmar1* expression (red-brown) is shown to be in cells that have ingressed into the blastocoel from an ectopic position relative to the endogenous vegetal plate (*endo16* stain at the bottom of Fig. 5G). This phenotype is observed in 88% of the injected embryos. Embryo detection of RNA by in situ hybridization was about the same as direct observation of the GFP (88 vs 86% of embryos expressing extra mesenchyme cells). Adjacent to the ectopic *gfp/pmar1* cells, ectopic *endo16* expression (dark blue-purple) is seen in an ectopic patch of cells (arrowhead in Fig. 5G), with the endogenous patch of *endo16* seen at the bottom (75% of the scored double injected embryos show adjacent patches of cells positive for *endo16* expression). Embryos injected with *HE-gfp* alone show the normal vegetal plate expression of *endo16* and nonmesenchyme patches of *gfp*-expressing cells in 98% of the cases (Fig. 5F). We conclude from these two experiments that *pmar1* is upstream of the ES.

Pmar1 expression is upstream of secondary endomesoderm induction

In the classic Hörstadius experiment, a second gut is induced if micromeres are transplanted to the animal pole of the sea urchin embryo (Hörstadius, 1939; Ransick and Davidson, 1993). Given that Pmar1 rescues the early specification of micromeres after removal of β -catenin from nuclei and Pmar1 controls expression of the two induction signals, we next asked if SoxB1 clearance occurs in the animal pole in response to *pmar1* expression by transplanted micromeres or mesomeres. Micromeres pretreated in several ways were transplanted to the animal poles of early fourth cleav-

age hosts. Control micromeres induced the secondary axis as expected. Micromeres expressing Δ -*cadherin* failed to induce a secondary axis when transplanted to the animal pole as shown earlier (Logan et al., 1999). If normal micromeres or Δ -*cadherin*-expressing micromeres also expressing *pmar1* are placed at the animal pole, the host cells nearby eliminate SoxB1 (Fig. 6). Similarly, mesomeres expressing *pmar1* have the ability to induce SoxB1 nuclear elimination if placed at the animal pole of control embryos (Fig. 6). We conclude that *pmar1* expression is upstream of a signal that induces ectopic clearance of SoxB1 from cells at the animal pole. It is likely that this signal and its response are at least a portion of secondary axis induction, a test for which we have a molecular assay. Most embryos under these conditions go on to make ectopic skeletons and a secondary gut, indicating that *pmar1* expression provides micromeres or mesomeres with the capability to induce a secondary axis.

Discussion

Use of blastomere transplantation to test gene regulatory network logic

The GRN for micromere specification and function published by Oliveri et al. (2002) was based entirely on whole embryo data. Several kinds of experimental information underlie the provisional GRN. The most basic is the time and place of expression of all the genes that participate in the network. Causal relationships within the GRN emerged from extensive QPCR observations on the specific consequences to other genes after perturbing regulatory genes specifically in micromeres and their descendants. WMISH experiments were carried out to reveal the effects on other genes of the spatial misexpression of *pmar1*. The *pmar1* gene encodes a transcriptional repressor normally active only in micromeres. Its function is proposed to inactivate an otherwise globally active repressor(s), thereby allowing micromere-specific genes to be expressed only in that lineage. To those data, the experiments in this manuscript add direct biological tests of predictions of the GRN. The correct placement of three major functions of *pmar1* in micromeres is supported in micromere transplantation experiments. Further, ectopic activation of *pmar1* transfates other cells of the embryo to carry out the micromere program showing that expression *pmar1* is sufficient for those cells to become PMCs.

Fig. 7 is a current version of the micromere specification GRN, modified somewhat from that shown in Oliveri et al. (2002), and simplified to focus on the results of tests in the present set of experiments. None of the other modifications, which are based on more recent data (<http://www.its.caltech.edu/~mirsky/qper.htm>), affect any aspects of the GRN directly relevant to the present work. The GRN is essentially a logic map, intended to display the regulatory

activators that account for the specific functions executed by the micromere lineage up to the point of ingression (20 h in *S. purpuratus*). There are three such functions: the expression of the “early signal” (ES) required for *veg2* endomesoderm specification; the subsequent expression of the Delta signal required for specification of pigment and other mesoderm cell types from *veg2* blastomeres lying adjacent to the micromeres; and the installation of a skeletogenic regulatory state in the micromeres.

The several specific features of the GRN shown in Fig. 7 that were directly challenged by the experiments in this manuscript are: (1) the prediction that expression of the *pmar1* gene is all that is required to transduce the essential β -catenin input into the micromere specification system; (2) the prediction that expression of the *pmar1* gene is all that is required to cause any cell in the embryo to execute all three of the functions normally carried out by the micromere lineage; (3) the corollary prediction that no prelocalized micromere “factors” or other features are needed downstream of *pmar1* gene for it to execute its role in micromere specification, nor are there any signals from the *veg2* cells adjacent to the micromeres required; and (4) the prediction implicit in the GRN of Fig. 7 that, once *pmar1* is expressed, micromere specification carries on autonomously.

Tests of the predictions

The experiments of Fig. 1 show that expression of *pmar1* in micromeres alone suffices to rescue skeletogenic specification dependent on the β -catenin input. The experiment in Fig. 2 generalizes this same result to blastomeres normally fated to become ectoderm (mesomeres). Therefore, *pmar1* expression suffices to produce the skeletogenic regulatory state, and its downstream effector functions, in cells of any lineage, position, and (normally) fate. This means that there can be no requirement for any other maternal micromere-specific factors downstream of *pmar1* itself.

In the experiments of Figs. 1–3, pigment cells are formed in the host embryos from host embryo precursors. This induction depends on expression of *pmar1* (i.e., presence of *pmar1* mRNA) in transplanted micromeres also bearing Δ -cadherin (Figs. 1 and 3). No pigment cells are found if the transplanted micromeres express Δ -cadherin mRNA alone or if the micromeres are simply removed from an otherwise normal embryo. Mesomeres expressing *pmar1* also suffice to induce pigment cells if transplanted to a micromereless host. Therefore, as Fig. 7 predicts, *pmar1* expression suffices to permit inductively functional Delta expression, even in mesomeres. The dependence of *delta* expression in micromeres on β -catenin nuclearization is therefore due entirely to the fact that the β -catenin input is needed for *pmar1* expression (Oliveri et al., 2002).

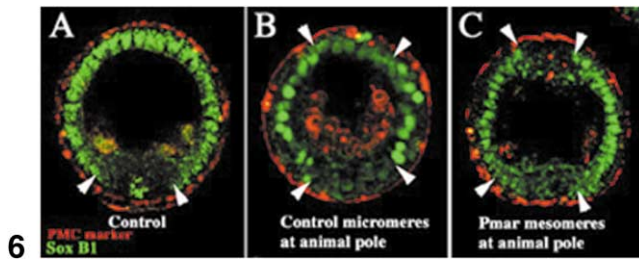
There is extensive evidence that *delta* expression lies downstream of *pmar1*. It cannot lie upstream because the *delta* gene is expressed much later than is *pmar1*. Nor is the *delta* gene wired in parallel with *pmar1* with independent

inputs because perturbation of *pmar1* expression specifically affects *delta* expression in the embryo and also at the *cis*-regulatory level (Oliveri et al., 2002; unpublished data from our laboratory). Nor can *delta* be upstream of the ES because this signal is passed at a stage prior to the time when the *delta* gene is activated (Ransick et al., 1995). A recent experiment of Sweet et al. (2002) shows that *delta* expression by mesomeres endows these cells with the ability to generate endoderm and skeleton, as well as mesoderm. However, blockade of *delta* translation with a Morpholino antisense oligo shows that, in a normal context, both skeleton formation and endoderm specification proceed normally (Sweet et al., 2002). The animal cap experiment thus indicates a train of regulative respecifications, and does not in fact conflict with the evidence underlying the present experiment, which places *pmar1* upstream of *delta*, and *delta* specifically responsible for the induction of pigment cells (Oliveri et al., 2002; Davidson et al., 2002; Sweet et al., 2002).

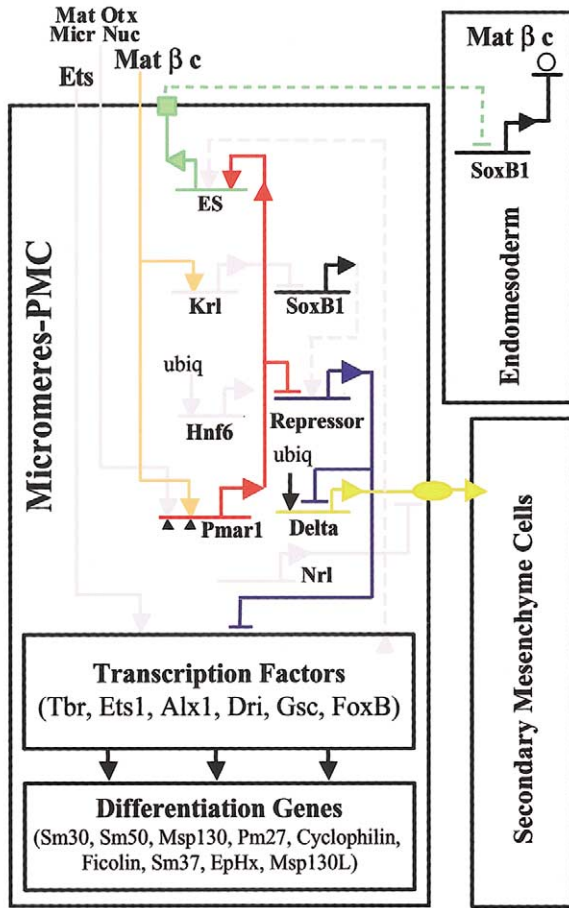
Figs. 4–6 concern the dependence of the ES on *pmar1* expression, also indicated in Fig. 7. Clearance of SoxB1 from endomesoderm nuclei is used as an early index of the effect of the ES on the *veg2* endomesodermal precursors (unpublished data). Fig. 4 shows that this clearance fails in micromereless embryos and in embryos to which micromeres expressing Δ -cadherin mRNA are transplanted. But again, transplanted micromeres expressing both Δ -cadherin mRNA and *pmar1* mRNA lead to SoxB1 clearance in the adjacent cells (Fig. 4), and a normal archenteron is also induced to form. Very dramatically, SoxB1 clearance can even be induced ectopically by transplanting mesomeres expressing *pmar1* mRNA to the animal pole (Fig. 6). In Fig. 5, it is similarly demonstrated that *endo16*, an endomesodermal marker gene, is activated in mesomeres adjacent to clones of cells producing Pmar1 from a genetic expression construct. The ES mRNA is therefore also likely to be an early transcriptional product of the micromeres. Combined, these experiments show that expression of the ES in any cell requires *pmar1* expression; that the need for the β -catenin input for ES expression is again by way of the *pmar1* gene; and that no micromere-specific features downstream of *pmar1* expression are required for ES emission.

The micromere GRN

From these experiments, it can be concluded that no additional inputs downstream of *pmar1* gene expression are exclusive to micromeres. This means that the *cis*-regulatory element of *pmar1* is almost certainly the furthest upstream zygotic transcriptional apparatus in the micromere specification GRN. This gene is activated immediately upon the birth of the micromeres (Oliveri et al., 2002), so temporal considerations make it extremely unlikely that there is any additional micromere-specific zygotic transcriptional function upstream of it. The *pmar1 cis*-regulatory element is already in hand, and its inputs will soon be known. The



6



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Fig. 6. *Pmar1*-expressing micromeres or mesomeres have the capacity to induce a secondary axis if transplanted to the animal pole. (A) The vegetal pole of control embryos clears SoxBI due to the presence of micromeres [now visible as PMCs (red) in the blastocoel (6/6 embryos examined)]. (B) If control micromeres are placed at the animal pole of a control embryo, they induce ectopic nuclear clearance of SoxBI (6/6 embryos examined). (C) That same ectopic SoxBI clearance is present at the animal pole if mesomeres expressing *pmar1* are transplanted to the animal pole (4/6 embryos examined). Green is immunostaining for SoxBI, and red is a specific PMC antibody. The hyaline layer outside the embryo is also stained nonspecifically with the red antibody (see Materials and methods for details).

Fig. 7. Micromere specification gene regulatory network. The network shown is modified from Oliveri et al. (2002). The diagram represents the “view from the genome”: all the known interactions at gene level relevant for the micromeres-PMC specification. The colored lines indicate the interactions analyzed in this paper; the gray lines are not considered here. More details are available in the Web site: <http://www.its.caltech.edu/~mirsky/endomes.htm>. Arrows indicate activating positive functions and bars repressive interactions. The *pmar1* gene (red) is depicted in the middle

β -catenin/TCF input is surely among them, as most likely is an Otx input (Davidson et al., 2002b; cf. QPCR data at <http://www.its.caltech.edu/~mirsky/qpcr.htm>). When completed, the specific inputs to which the *pmar1* cis-regulatory element responds will define in molecular terms the maternal components localized in micromeres after the unequal 4th cleavage that are responsible for triggering activation of the micromere GRN.

Additional evidence that lies outside the scope of this paper explicitly places all the known skeletogenic subnetwork regulators, i.e., *tbr*, *ets*, *alx1*, *dri*, and *foxb*, downstream of the *pmar1* derepression system (QPCR perturbation experiments, *op cit.*; Amore et al., 2003; and much unpublished additional data of P.O. and E.H.D.). Thus, overexpression or knockout of these regulators affects skeletogenic functions (for *ets*, Kurokawa et al., 1999; for *dri*, Amore et al., *op. Cit.*; for *tbr*, Davidson et al., 2002a and Croce et al., 2001; plus other unpublished data). But these perturbations do not affect expression of the ES or the Delta signal, for example, as has been shown clearly in each case by morpholino knockouts followed by measurement of *delta* transcript level (data on QPCR web site, *op cit.*). As illustrated in Fig. 7, this is because the *delta* gene and the skeletogenic regulatory apparatus are hooked up in parallel to the *pmar1* derepression system.

A final point that emerges into the foreground of this work is that the GRN affords a direct, two-way transit between the evidential world of experimental embryology and the sequence-based logic relationships wired into the genomic regulatory system. This is of great interest for the design of future research. On the one hand, it breathes conceptual import into what has been a largely empirical branch of classical embryology. On the other hand, it provides a vivid means of testing the completeness of the logic predictions of the GRN, where the readout can be perceived directly, at the level of cell fate and function.

of the GRN and is activated by maternal β -catenin (orange) and maternal Otx. *Pmar1* is modeled to repress an unknown ubiquitous repressor(s) (blue). That repressor(s) prevents the micromere specification pathway from moving forward to activate specific early micromere-PMC transcription factors (*tbr*, *ets*, *alx1*, *dri*, and *foxb*). These, acting in different combinations, are responsible for the expression of the skeletogenic differentiation genes. The repressor also blocks the expression of *delta* (yellow), schematically represented as a membrane protein. The Delta ligand is responsible, through an interaction with Notch (yellow arrow), for the specification of the secondary mesenchyme cells (lower box on the right; Sherwood and McClay 1999; Sweet et al., 2002). When *Pmar1* silences the repressor(s), *delta* is expressed and the SMC specification pathway is activated. *Pmar1* also activates the production of the ES, illustrated as embedded in the schematic membrane (green). The double arrow from *pmar1* to the unknown gene coding for the ES indicates a possible indirect interaction. So far, *Pmar1* has been shown to work as a repressor (Oliveri et al., 2002). The ES function is indirectly connected (green dashed line) to SoxBI (black) clearance from the endomesoderm territory (upper right box). Thus, all three known early functions of the micromeres are enabled by *Pmar1* expression.

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