Gene regulatory networks and developmental plasticity in the early sea urchin embryo: alternative deployment of the skeletogenic gene regulatory network

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Cell fates in the sea urchin embryo are remarkably labile, despite the fact that maternal polarity and zygotic programs of differential gene expression pattern the embryo from the earliest stages. Recent work has focused on transcriptional gene regulatory networks (GRNs) deployed in specific embryonic territories during early development. The micromere-primary mesenchyme cell (PMC) GRN drives the development of the embryonic skeleton. Although normally deployed only by presumptive PMCs, every lineage of the early embryo has the potential to activate this pathway. Here, we focus on one striking example of regulative activation of the skeletogenic GRN; the transfating of non-skeletogenic mesoderm (NSM) cells to a PMC fate during gastrulation. We show that transfating is accompanied by the de novo expression of terminal, biomineralization-related genes in the PMC GRN, as well as genes encoding two upstream transcription factors, Lvalx1 and Lvtbr. We report that Lvalx1, a key component of the skeletogenic GRN in the PMC lineage, plays an essential role in the regulative pathway both in NSM cells and in animal blastomeres. MAPK signaling is required for the expression of Lvalx1 and downstream skeletogenic genes in NSM cells, mirroring its role in the PMC lineage. We also demonstrate that Lvalx1 regulates the signal from PMCs that normally suppresses NSM transfating. Significantly, misexpression of Lvalx1 in macromeres (the progenitors of NSM cells) is sufficient to activate the skeletogenic GRN. We suggest that NSM cells normally deploy a basal mesodermal pathway and require only an Lvalx1-mediated sub-program to express a PMC fate. Finally, we provide evidence that, in contrast to the normal pathway, activation of the skeletogenic GRN in NSM cells is independent of Lvpmar1. Our studies reveal that, although most features of the micromere-PMC GRN are recapitulated in transfating NSM cells, different inputs activate this GRN during normal and regulative development.

KEY WORDS: Sea urchin embryo, Pattern formation, Gene regulatory networks, Primary mesenchyme, Transfating, Regulative development

INTRODUCTION

Early experimental embryological studies first revealed the striking regulative properties of sea urchin embryos (Driesch, 1892; Höristadius, 1939). Blastomere isolation and recombination experiments demonstrated that the fates of cells in the early embryo are not rigidly fixed, but are influenced by signals from neighboring cells. This developmental plasticity seems at odds, however, with evidence that: (1) cell fates are biased at early stages; (2) embryonic patterning is entrained by molecular asymmetries within the unfertilized egg; and (3) distinct domains of differential gene expression arise very early in development. The classical embryological investigations that revealed the regulative properties of cleavage-stage embryos also demonstrated biases in the developmental programs of blastomeres. Other studies have shown that the unfertilized egg is polarized along the animal-vegetal (AV) axis (reviewed by Ettensohn and Sweet, 2000; Brandhorst and Klein, 2002; Angerer et al., 2002; Oliveri et al., 2003; Yamazaki et al., 2005). The localization of maternal regulators of canonical Wnt signaling is asymmetrical, and its partner, LEF-TCF, are required for the expression of pmar1, the earliest zygotically expressed component of the micromere-PMC GRN (Kitamura et al., 2002; Nishimura et al., 2004; Oliveri et al., 2002; Oliveri et al., 2003; Yamazaki et al., 2005). pmar1 is
probably activated directly by β-catenin (Nishimura et al., 2004) and appears to be the only critical target of this protein with respect to PMC specification (Oliveri et al., 2003).

Pmar1 is a transcriptional repressor and activates downstream genes in the PMC GRN by a double-repression mechanism (Oliveri et al., 2002). Early targets include genes encoding the transcription factors Alx1 (Ettenson et al., 2003), Ets1/2 (Kurokawa et al., 1999) and Tbr (Fuchikami et al., 2002). alx1, which encodes a Paired-class homeodomain-containing protein, is expressed in the large daughter cells of the micromeres (the founder cells of the PMC lineage) as soon as they are born. alx1 is required for PMC ingestion and skeletogenesis, and regulates these behaviors via downstream targets that include snail (Wu and McClay, 2007) and deadringer (Amore et al., 2003). Terminal genes in the PMC network encode proteins that directly regulate the morphogenetic behaviors of the cells, including ingestion, migration, fusion and skeletogenesis. Of these, the best understood are biomineralization genes, many of which have now been identified through genome-wide analysis (Livingston et al., 2006; Wilt and Ettenson, 2007).

Although the skeletogenic GRN is normally deployed only in the prospective PMCs, a striking feature of sea urchin development is that every lineage of the early embryo has the capacity to activate this GRN. A variety of surgical and molecular manipulations have revealed this developmental plasticity. For example, removal of micromeres at the 16-cell stage results in transformation of macromere-derived cells to a skeletogenic fate (Hörstadius, 1939; Sweet et al., 1999). Even animal blastomeres can be induced to activate the skeletogenic GRN by treating the cells with LiCl (Livingston and Wilt, 1989), by misexpressing Pmar1 or the signaling molecule Delta (Oliveri et al., 2002; Sweet et al., 2002) or by exposing animal blastomeres to inductive signals from micromeres (Minokawa et al., 1997). Remarkably, some populations of cells retain the capacity to activate the PMC GRN even after the onset of gastrulation. Microsurgical removal of PMCs at the early gastrula stage causes a subpopulation of non-skeletogenic mesoderm (NSM) cells to switch to the PMC fate (Ettenson and McClay, 1988; Ettenson, 1990; Ettenson, 1992). Surgical removal of both PMCs and NSM cells at the archenteron tip leads to fate-switching to PMCs and NSM cells at the archenteron tip (Ettensohn and McClay, 1988; Ettensohn, 1990; Ettensohn, 1992). Surgical removal at the mesenchyme blastula stage, and were raised continuously in the dark in the presence of the drug. Embryos were fixed for 1 hour at room temperature in freshly prepared 20% (vol/vol) formalin (37% formaldehyde solution) in seawater (SW) and then permeabilized in 100% methanol for 10 minutes at –20°C. Fixed embryos were stored overnight at 4°C in 70% ethanol before being processed further.

Microinjection of morpholino oligonucleotides (MOs), mRNAs and fluorescent dextrans

Microinjection of MOs and mRNAs into fertilized eggs was carried out as described by Cheers and Ettenson (Cheers and Ettenson, 2004). LvAlx1 MO was described previously (Ettenson et al., 2003). MO injection solution contained 2–4 mM LvAlx1 MO, 20% glycerol and 0.1% Rhodamine dextran in water. mRNAs were injected at 0.1-4.0 mg/ml in 20% glycerol. mRNA injection solutions were prepared using RNase-free water. Microinjection of fluorescent dextran (5% lysine-fixable fluorescein dextran, M₄=10,000; Invitrogen) into micromeres was carried out according to the method of Ruffins and Ettenson (Ruffins and Ettenson, 1996).

RESULTS

Activation of the micromere-PMC GRN during NSM transfecting

Transfected NSM cells exhibit many properties normally restricted to PMCs, including (1) the ability to respond to PMC-specific guidance cues, (2) the production of signals that suppress NSM transfecting and (3) the formation of an endoskeleton. We previously used a monoclonal antibody to show that transfected cells express PMC-specific proteins of the MSP130 family (Ettenson and McClay, 1988).

To show directly that other downstream, skeletogenic genes in the PMC GRN are activated during transfecting, we analyzed the expression of Lvsp16 and Lvsp50a following PMC removal. LvsSM30 and LvsSM50 are secreted proteins occluded within the calcified spicule (Wilt and Ettenson, 2007). LvsP16 and LvsP50a are novel, PMC-specific transmembrane proteins (Illies et al., 2002) (C.A.E., unpublished observations). LvsP16 plays an essential role in skeletal rod elongation (Cheers and Ettenson, 2005). LvsP50a was identified through an in situ hybridization screen and its function is unknown. Each of the four mRNAs is expressed specifically by cells of the large micromere-PMC lineage during normal development, as assessed by WMISH.

MATERIALS AND METHODS

Embryo culture

Adult Lytechinus variegatus were obtained from the Duke University Marine Laboratory (Beaufort, NC, USA) and Carolina Biological Supply (Burlington, NC, USA). Collection of gametes and embryo culture were carried out as described previously (Cheers and Ettenson, 2005).

Microsurgery

Blastomere isolation and recombination experiments were performed as described by Sweet et al. (Sweet et al., 2004). Animal caps were obtained from 16-cell stage embryos. PMCs were removed from mesenchyme blastula stage embryos following the method of Ettenson and McClay (Ettenson and McClay, 1988).

Whole-mount in situ hybridization (WMISH)

WMISH was carried out according to the method of Zhu et al. (Zhu et al., 2001) with the modification that formalin was typically used as the fixative instead of glutaraldehyde. Formalin-fixed specimens were fragile and usually exhibited little background staining. Embryos were fixed for 1 hour at room temperature in freshly prepared 20% (vol/vol) formalin (37% formaldehyde solution) in seawater (SW) and then permeabilized in 100% methanol for 10 minutes at –20°C. Fixed embryos were stored overnight at 4°C in 70% ethanol before being processed further.
Lvsm30, Lvsm50, Lvp16 and Lvp58α mRNAs accumulated in NSM cells during transfating (Fig. 1). Moreover, at late developmental stages, levels of Lvsm30, Lvsm50 and Lvp16 mRNAs were spatially regulated within the skeletogenic syncytium in a pattern that appeared identical to that normally exhibited within the PMC syncytium (Guss et al., 1997; Illies et al., 2002). For example, Lvsm30 mRNA was expressed at high levels in the ventrolateral PMC clusters at the late gastrula stage but at much lower levels in the ventral region during transfating (Fig. 1C). Moreover, at late stages of PMCs, LVSM30 was expressed at high levels in the ventral PMC chain (Fig. 1C). Lvsm50 was expressed at high levels in the somatic region at the late gastrula stage.

We next examined the expression of upstream components in the micromere-PMC GRN. Initially, we focused on three well-characterized genes that encode transcription factors; ets1/2, tbr and alx1. We previously cloned and characterized alx1 from L. variegatus (Ettenson et al., 2003) and in the course of this study cloned Lvtbr and Lvets/2. Lvalx1 is expressed only by cells of the large micromere-PMC lineage (Ettenson et al., 2003) and we confirmed that Lvtbr expression is also restricted to PMCs (C.A.E., unpublished observations). ets1/2 mRNA has been reported to be restricted to PMCs in Hemicentrotus pulcherrimus (Kurokawa et al., 1999) but is expressed by both PMCs and NSM cells in Paracentrotus lividus (Rottinger et al., 2004) and Strongylocentrotus purpuratus (Rizzo et al., 2006). We found that, in L. variegatus, ets1/2 mRNA accumulates in both PMCs and NSM cells and we used a polyclonal antiserum to show that LVETS1/2 protein accumulates in the nuclei of both cell types (data not shown). Because these experiments showed that NSM cells normally express LVTBR during gastrulation, we focused on the possible activation of the other two genes, Lvalx1 and Lvtbr during transfating.

Expression of both Lvalx1 and Lvtbr was detected in transfating cells after microsurgical removal of PMCs (Fig. 2). WMISH signal was more robust for Lvalx1 and it was therefore possible to detect Lvalx1 mRNA at much earlier stages of transfating. Lvalx1 mRNA was first detectable in cells near the tip of the archenteron 3-4 hours after PMC removal (Fig. 2C). In many embryos, it appeared that Lvalx1 expression was not radially symmetrical around the gut but was concentrated on one side (Fig. 2D,F). This was not observed in all cases, but may have been obscured in some embryos because of their orientation. Lvtbr mRNA was more difficult to detect by in situ hybridization, presumably because this mRNA was not as abundant as Lvalx1 mRNA. Nevertheless, 9-10 hours after PMC removal, it was clear that Lvtbr mRNA had accumulated in transfated mesenchyme cells that were arranged in a ring pattern characteristic of PMCs (Fig. 2H).
Alx1 is required in transfating cells

We next asked whether Lvalx1 function was required to activate the skeletogenic GRN in NSM cells. We showed previously that injection of Alx1 morpholino (MO) into fertilized eggs completely blocked the formation of skeletal elements (Ettensohn et al., 2003). This indicated that NSM transfating did not occur in LvAlx1 MO-injected embryos, but did not prove that this was a direct consequence of blocking LvAlx1 expression in NSM cells. In particular, we could not rule out the possibility that the signal produced by large micromere descendants that suppresses NSM transfating might still be transmitted between the two cell populations in Alx1 MO-injected embryos.

We injected LvAlx1 MO into fertilized eggs and allowed them to develop to the 16-cell stage, when the quartet of micromeres was removed (Fig. 3A). Previous studies showed that removal of micromeres results in a robust transfating response (Sweet et al., 1999). In control experiments, we confirmed that >90% of micromere-deficient embryos produced a normally patterned skeleton (19/21 cases; Fig. 3F,G). Micromere-deficient, Alx1 MO-injected embryos, however, showed no transfating response even after extended culture periods (2-3 days). Micromere-deficient, LvAlx1 MO-injected embryos gastrulated and gave rise to various NSM derivatives, but almost no 6a9-positive cells formed in these embryos, which also failed to form skeletal elements (67/70 cases) (Fig. 3H,I). LvAlx1 MO was present in mesomeres (in addition to macromeres) in this experiment, but Lvalx1 is not expressed in mesomere progeny following PMC removal (Fig. 2). Therefore, these experiments indicate that Lvalx1 function is required autonomously in macromere-derived cells for activation of the skeletogenic GRN.

We also tested whether Lvalx1 function was required for activation of the micromere-PMC GRN in animal blastomeres (Fig. 4). Mesomeres give rise to skeletogenic cells when treated with LiCl (Livingston and Wilt, 1989). LiCl probably exerts this effect by stabilizing β-catenin (Kao and Elinson, 1998; Logan et al., 1999).

We confirmed that animal caps isolated from 16-cell stage embryos and cultured in seawater gave rise only to ectoderm (Fig. 4B,E), When animal caps were treated with 50 mM LiCl for 3 hours immediately after isolation, however, they usually gave rise to gastrulae and 63% of these embryos (92/146 cases) eventually formed normally patterned skeletons (Fig. 4C,F). LiCl treatment also induced the expression of Lvalx1 and Lvp16, a target of Lvalx1 (Cheers and Ettensohn, 2005), in isolated animal caps (Fig. 4H). In contrast, animal caps isolated from LvAlx1 MO-injected embryos did not give rise to 6a9-positive cells or skeletal elements when exposed to LiCl under identical conditions (56/57 cases), although LiCl still induced the formation of guts in ~70% of the embryos (Fig. 4D,G). These findings demonstrate that Lvalx1 is required for ectopic activation of the PMC GRN in animal blastomeres.

Alx1 regulates PMC ingestion and the activation of skeletogenic genes (Ettensohn et al., 2003). We asked whether the PMC-derived signal that suppresses NSM transfating might also be regulated by alx1 (Fig. 5). Recombinant embryos were produced by grafting micromere quartets to animal caps isolated from 16-cell stage embryos (Fig. 5A). Previous studies showed that micromeres induce animal blastomeres to give rise to vegetal cell types, including cells that transfate to a skeletogenic phenotype upon removal of the micromere descendants (Minokawa et al., 1997). Micromere quartets were isolated from embryos that had been injected with fluorescent dextran alone (controls), or co-injected with fluorescent dextran and LvAlx1 MO. In control embryos, fluorescent dextran-labeled micromeres gave rise to PMCs and these formed extensive skeletal elements (16/17 cases; Fig. 5B,E). When the micromeres contained LvAlx1 MO, however, their descendants remained associated with the tip of the archenteron or ingressed into the blastocoel (Fig. 5C,F). Large numbers of 6a9-positive cells formed...
in these embryos and gave rise to skeletal elements (Fig. 5D,G), but these cells were not labeled with fluorescent dextran and were therefore derived exclusively from the animal cap (15/19 cases). These experiments demonstrate that the ability of micromere progeny to suppress NSM transfating is dependent on Lvalx1 function. One possibility is that the gene encoding the PMC-derived signaling molecule is regulated (directly or indirectly) by Lvalx1.

Alternatively, Lvalx1 may control aspects of the motility or morphogenesis of the large micromere descendants that mediate signaling. For example, ingression and filopodial extension by the PMCs might be required for signaling. Although many micromere progeny ingress even when Lvalx1 expression is blocked (Fig. 5C,F), their migration is delayed.

The MAPK pathway is required for transfating
The MAPK pathway plays an essential role in PMC specification (Fernandez-Serra et al., 2004; Rottinger et al., 2004). Treatment of embryos with U0126, a MEK inhibitor, blocks PMC specification in both S. purpuratus and P. lividus. Overexpression of a dominant negative form of MEK produces a similar phenotype.

We confirmed that treatment of L. variegatus embryos with U0126 suppressed PMC formation (Fig. 6A-F). Continuous exposure of L. variegatus embryos to U0126 (6-25 μM) from the 2-cell stage blocked PMC ingression and greatly reduced the numbers of 6a9-positive cells at the late gastrula stage (controls: 68.8±16.6, mean ± s.d., n=30; U0126-treated: 1.3±2.7, n=300). U0126 treatment also blocked skeletogenesis, as reported previously. Some batches of embryos treated with a relatively high concentration of U0126 (25 μM) exogastrulated, whereas at lower concentrations the archenteron invaginated, albeit in a delayed fashion. The reduction in 6a9-positive cells was not due simply to a delay in PMC
specification, as numbers of 6a9-positive cells in U0126-treated embryos remained very low even when embryos were cultured for 2-3 days.

To test whether MAPK signaling was required for transfating, we removed PMCs at the mesenchyme blastula stage and then transferred the embryos to 6 μM U0126. As a control, sibling PMC-deficient embryos were allowed to continue development in normal seawater. In untreated control embryos, NSM cells converted to the PMC fate and synthesized a correctly patterned skeleton (Fig. 6G-I). By contrast, in sibling U0126-treated, PMC-deficient embryos, no 6a9-positive cells formed and skeletogenesis was completely suppressed (Fig. 6J-L). U0126 also completely blocked expression of Lvalx1 in PMC-deficient embryos, as assessed by WMISH (Fig. 6H,K). Other morphogenetic processes, including archenteron invagination and compartmentalization, invagination of the stomodeum, and ciliary band formation, took place normally in U0126-treated, PMC-deficient embryos. These experiments show that MAPK signaling is required in transfating NSM cells to activate the expression of Lvalx1 and downstream skeletogenic genes, mirroring the role of this pathway in normal development.

Overexpression of Lvalx1 is sufficient to induce transfating of macromere-derived cells
Several genes in the PMC GRN are expressed by NSM cells during gastrulation (see Discussion). Because Lvalx1 is an exception and a critical component of the network, we tested whether ectopic expression of this gene might be sufficient to convert NSM cells to a skeletogenic fate.

We overexpressed Lvalx1 throughout the embryo by microinjecting mRNA into fertilized eggs. Overexpression of Lvalx1-GFP confirmed that the protein was localized in the nuclei of all cells, including cells within the vegetal plate (Fig. 7A,B). Embryos injected with wild-type Lvalx1 (Lvalx1.WT) mRNA (unteagged) at concentrations of 0.4-0.75 mg/ml showed a reproducible increase in numbers of 6a9-positive cells when examined at the late gastrula stage (Fig. 7C-E, Fig. 8). In some cases, very large numbers of 6a9-positive cells were observed (>100 cells). The 6a9-positive cells were usually arranged in a radially symmetrical, circumferential belt around the equator and the vegetal pole. These cells also expressed Lup16 (Fig. 7F,G). Injection of higher concentrations of Lvalx1 mRNA (1.5 mg/ml or higher)
delayed development and resulted in decreased numbers of 6a9-positive cells, whereas concentrations below 0.1 mg/ml had no effect on development. In control experiments, we injected 20% glycerol (the mRNA carrier solution) or similar concentrations of a mutant form of Lvalx1 mRNA with a stop codon introduced immediately upstream of the homeodomain (Lvalx1.STOP). This mRNA encoded a short (115 aa) N-terminal fragment of LvAlx1 that cannot bind to DNA. Neither reagent caused an increase in 6a9-positive cells (Fig. 8). Although almost all uninjected control embryos and embryos injected with Lvalx1.STOP (31/32) formed only two tri-radiate spicule rudiments, embryos injected with Lvalx1 mRNA usually produced supernumerary tri-radiate spicules (average=4.6/embryo, n=28).

To confirm that the increase in 6a9-positive cells in Lvalx1 mRNA-injected embryos resulted from transfating of macromere progeny rather than proliferation of PMCs, we carried out lineage tracing experiments (Fig. 9A). Fertilized eggs were injected with Lvalx1.WT (0.38 mg/ml) and at the 16-cell stage, one macromere was injected with fixable, fluorescein dextran. At the gastrula stage, embryos were fixed and processed for immunostaining using mAb 6a9 and a Cy5-conjugated anti-mouse secondary antibody. Analysis of dextran-labeled embryos by confocal microscopy confirmed that overexpression of LvAlx1 caused transformation of macromere-derived cells to a skeletogenic fate (Fig. 9B,C).

**Lvpmar1 does not activate the skeletogenic GRN network during NSM transfating**

*pmar1* is a critical, upstream component of the large micromere-PMC GRN (Kitamura et al., 2002; Nishimura et al., 2004; Oliveri et al., 2002; Oliveri et al., 2003; Yamazaki et al., 2005). Because we found that at least two genes encoding transcription factors of the PMC GRN, Lvalx1 and Lvtbr, are expressed ectopically in macromere-derived cells during transfating, we asked whether these genes might be activated by *pmar1*.

Preliminary studies confirmed that *pmar1* plays a role in PMC specification in *L. variegatus* similar to that observed in other species. Analysis of a BAC clone containing the *Lvpmar1* locus (GenBank accession no. AC131562, Sea Urchin Genome Project BAC Clone #170H13) showed that in *L. variegatus*, as in other species, the *pmar1* locus consists of several tandem copies of the gene (Fig. 10A). At least ten repeats of the gene are present in *L. variegatus*, and possibly more, as one end of the BAC insert lies within the tenth repeat. The coding sequences of the tandem copies of *Lvpmar1* are very similar at the nucleotide level and we designed a single pair of PCR primers that would recognize transcripts from any of the ten genes. These primers spanned a conserved intron, thereby allowing us to unambiguously distinguish mRNA-based amplification from...
amplification driven by contaminating genomic DNA. PCR amplification using these primers yielded a single product of the expected size. This PCR product was cloned and sequenced, confirming that it corresponded to Lvpmar1 (data not shown).

Developmental RT-PCR analysis showed that Lvpmar1 was expressed in a temporal pattern similar to that observed in other species (Fig. 10B). Lvpmar1 mRNA was not detectable in unfertilized eggs but was expressed transiently during cleavage. Injection of Lvpmar1 mRNA into fertilized eggs resulted in a dramatic transformation of most cells of the embryo to a skeletogenic fate (Fig. 10C), consistent with findings in S. purpuratus. These findings suggest that pmar1 has a similar developmental function in all three species and that the recruitment of this gene into the PMC GRN predated the split between the Strongylocentrotidae (S. purpuratus and H. pulcherrimus) and Toxopneustidae (L. variegatus), which occurred at least 45 million years ago (Smith et al., 2006).

We next explored whether Lvpmar1 expression was activated in NSM cells during transfating (Fig. 10D). PMCs were removed from mesenchyme blasula stage embryos and embryo lysates were prepared 0, 3, 6 and 9 hours after surgery. Lvalx1 and Lvp16, both of which are downstream of pmar1 in the micromere-PMC GRN, are expressed at high levels in transfating cells within 9 hours after PMC depletion (Fig. 2C-G, Fig. 10D).

In three independent RT-PCR experiments carried out on different batches of PMC-deficient embryos, we were unable to detect Lvpmar1 expression (Fig. 10D). To assess the sensitivity of our RT-PCR analysis, we prepared cell lysates from normal 16-cell stage embryos, when Lvpmar1 is expressed at relatively low levels, and prepared serial dilutions of these cell lysates. We could reliably detect Lvpmar1 expression using quantities of cell lysate that were equivalent to 1/100 embryo (three independent replicates are shown in Fig. 10D). We cannot exclude the possibility that Lvpmar1 is expressed at very low levels in transfating NSM cells. These experiments indicate, however, that if pmar1 mRNA is present, it is expressed at a level less than 1% of that normally observed at the 16-cell stage.

**DISCUSSION**

**Activation of the skeletogenic GRN during normal and regulative development: different inputs result in the same output**

The findings reported here strongly suggest that the skeletogenic GRN is activated by distinct mechanisms during normal and regulative development (Fig. 11). During normal development, activation of the GRN in the micromere lineage requires the polarized nuclearization of β-catenin, a process entrained by the intrinsic AV polarity of the unfertilized egg. Unequal cell division may also play a role in the normal activation of the GRN, as pharmacological treatments that alter the position of the cleavage plane in vegetal blastomeres of 8-cell stage embryos also perturb PMC specification (Langlan and Whiteley, 1985). Blastomere isolation and transplantation experiments show that many aspects of the specification and early differentiation of micromeres, including the activation of several terminal skeletogenic genes in the PMC GRN, are independent of signals from other cells (Stephens et al., 1989; Stephens et al., 1990) (reviewed by Ettenshozn et al., 1997). Several workers have reported that micromeres isolated at the 16-cell stage and cultured in plain seawater (i.e. without serum or other supplements) execute many features of their normal morphogenetic program, including migration, fusion and the secretion of small calcareous granules (Hodor and Ettensohn, 1998; Okanewski, 1975; Page and Benson, 1992). It was therefore surprising when recent studies showed that the MAPK pathway plays an essential, positive role in the deployment of the PMC GRN, probably through a requirement for ERK-mediated phosphorylation of Ets1/2 (Fernandez-Serra et al., 2004; Rottinger et al., 2004). Two possible interpretations of these findings are that micromere descendants activate ERK via autocrine signaling or by a ligand-independent mechanism.

The regulation of the skeletogenic GRN in NSM cells during transfating appears different from the normal pathway in several respects. First, in NSM cells, the skeletogenic network is tightly regulated by extrinsic signals. These signals emanate from a different population of cells (PMCs) and, in contrast to possible homotypic interactions within the PMC lineage (Rottinger et al., 2004),
It will be important to determine whether hesC territory by a repressor of micromeres (Oliveri et al., 2002), which has recently been shown to be encoded by the gene (Revilla-i-Domingo et al., 2003). We therefore propose that in specific cellular contexts (e.g. perhaps in the context of a ‘mesenchymal’ GRN already deployed in NSM cells) Alx1 does not cause the kind of global transfecting response produced by Pmar1 (Oliveri et al., 2002; Oliveri et al., 2003). We have identified new examples of genes expressed only by these cell populations (Zhu et al., 2001) (C.A.E., unpublished observations). The cyIIa gene is expressed by PMCs and NSM cells (as well as endoderm cells) and a pan-mesodermal regulatory element has been identified (Martin et al., 2001). These observations suggest that NSM cells normally deploy many elements of the PMC GRN but lack certain key components that direct PMC fate specification.

One important consequence of PMC signaling is the repression of alx1 in NSM cells. Expression of this gene is sufficient to induce transfecting of macromere progeny (see below). At present, the link between the PMC-derived signal and the repression of alx1 in NSM cells is not understood. During normal development, alx1 and other early genes in the PMC GRN are thought to be repressed by the ‘repressor of micromeres’ (Oliveri et al., 2002), which has recently been shown to be encoded by the hesC gene (Revilla-i-Domingo et al., 2007). It will be important to determine whether hesC is normally expressed by NSM cells at the gastrula stage. If not, then this would imply that alx1 is normally repressed in the NSM territory by a hesC-independent mechanism or is no longer regulated by repression. If hesC is normally expressed by NSM cells and repressed during transfecting, then our findings suggest that such repression occurs by a pmar1-independent mechanism, in contrast to the normal pathway.

**Regulative deployment of the PMC GRN in different lineages is likely to be context dependent**

Several genes in the PMC GRN are expressed by NSM cells during normal development, including ets1/2 (Rottinger et al., 2004; Rizzo et al., 2006), snail (Wu and McClay, 2007) and delta (Sweet et al., 2002). NSM cells, like the presumptive PMCs, contain phosphorylated ERK (Fernandez-Serra et al., 2004; Rottinger et al., 2004); therefore, Ets1/2 is also likely to be phosphorylated in NSM cells. Other transcription factors have been identified that show restricted expression in PMCs and NSM cells (e.g. erg), and a recent in situ hybridization screen has identified new examples of genes expressed only by these cell populations (Zhu et al., 2001) (C.A.E., unpublished observations). The cyIIa gene is expressed by PMCs and NSM cells (as well as endoderm cells) and a pan-mesodermal regulatory element has been identified (Martin et al., 2001). These observations suggest that NSM cells normally deploy many elements of the PMC GRN but lack certain key components that direct PMC fate specification.

One critical component of the skeletogenic GRN not normally expressed by NSM cells is alx1 (Ettensohn and Ruffins, 1993). 2004), they act in a negative fashion to block the deployment of the skeletogenic GRN. In NSM cells, the skeletogenic GRN remains subject to cell signaling even late in development, after maternal regulators have presumably disappeared. The most direct evidence that control of the GRN differs during normal and regulative development comes from our observation that pmar1, a key activator of the micromere-PMC GRN, is not expressed at detectable levels by NSM cells during transfecting. Our findings therefore argue that different upstream mechanisms activate the skeletogenic GRN in micromeres and NSM cells, but result in the same output.

One important consequence of PMC signaling is the repression of alx1 in NSM cells. Expression of this gene is sufficient to induce transfecting of macromere progeny (see below). At present, the link between the PMC-derived signal and the repression of alx1 in NSM cells is not understood. During normal development, alx1 and other early genes in the PMC GRN are thought to be repressed by the ‘repressor of micromeres’ (Oliveri et al., 2002), which has recently been shown to be encoded by the hesC gene (Revilla-i-Domingo et al., 2007). It will be important to determine whether hesC is normally expressed by NSM cells at the gastrula stage. If not, then this would imply that alx1 is normally repressed in the NSM territory by a hesC-independent mechanism or is no longer regulated by repression. If hesC is normally expressed by NSM cells and repressed during transfecting, then our findings suggest that such repression occurs by a pmar1-independent mechanism, in contrast to the normal pathway.

**Regulative deployment of the PMC GRN in different lineages is likely to be context dependent**

Several genes in the PMC GRN are expressed by NSM cells during normal development, including ets1/2 (Rottinger et al., 2004; Rizzo et al., 2006), snail (Wu and McClay, 2007) and delta (Sweet et al., 2002). NSM cells, like the presumptive PMCs, contain phosphorylated ERK (Fernandez-Serra et al., 2004; Rottinger et al., 2004); therefore, Ets1/2 is also likely to be phosphorylated in NSM cells. Other transcription factors have been identified that show restricted expression in PMCs and NSM cells (e.g. erg), and a recent in situ hybridization screen has identified new examples of genes expressed only by these cell populations (Zhu et al., 2001) (C.A.E., unpublished observations). The cyIIa gene is expressed by PMCs and NSM cells (as well as endoderm cells) and a pan-mesodermal regulatory element has been identified (Martin et al., 2001). These observations suggest that NSM cells normally deploy many elements of the PMC GRN but lack certain key components that direct PMC fate specification.

One critical component of the skeletogenic GRN not normally expressed by NSM cells is alx1 (Ettensohn and Ruffins, 2003). Our experiments show that expression of Lvalx1 is sufficient to induce macromere descendants to activate downstream skeletogenic genes, including Lvp16 and genes of the msp130 family, and renders the cells responsive to PMC-specific guidance cues. Macromeres give rise to several cell types and we do not know which cells within this population respond to Lvalx1 misexpression. It is clear, however, that ectopic expression of Alx1 does not cause the kind of global transfecting response produced by Pmar1 (Oliveri et al., 2002; Oliveri et al., 2003). We therefore propose that in specific cellular contexts (e.g. perhaps in the context of a ‘mesenchymal’ GRN already deployed in NSM
cells), alx1 functions as a critical regulator of a molecular subroutine that provides inputs into several cell behaviors and activates the complete battery of skeletogenic genes.

Regulative deployment of the PMC GRN can occur in territories of the embryo other than the NSM. Mesomeres can be induced to activate the pathway by treating the cells with LiCl (Livingston and Wilt, 1989) or by ectopic activation of Notch-Delta signaling (Sweet et al., 2002). LiCl stabilizes β-catenin by inhibiting GSK3β and may activate pmar1 and the remainder of the PMC GRN by mechanisms that essentially mimic the normal upstream activation seen in the micromere-PMC lineage. This is consistent with the finding that misexpression of pmar1 alone in animal cells is sufficient to activate the PMC GRN (Olivieri et al., 2002; Olivieri et al., 2003). Activation of the skeletogenic GRN in mesomeres by ectopic activation of Notch-Delta signaling has not been investigated in detail. Activation of the skeletogenic GRN by presumptive endodermal cells during gastrulation (McClay and Logan, 1996) may occur by yet another mechanism, as these cells have their own distinctive developmental history. Further investigations of each of these regulative pathways will be informative and may reveal common features.

The PMC GRN and the evolution of regulative processes
It is widely believed that micromeres and an early-ingressing, skeletogenic mesenchyme are relatively recent evolutionary innovations. All adult echinoderms have a calcified endoskeleton, suggesting that this is an ancient feature of the phylum. Among echinoderms, however, only echinoids (sea urchins and sand dollars) and ophiuroids (brittle stars) form an extensive embryonic skeleton, and only echinoids form micromeres. Furthermore, the development of cidaroid urchins (subclass Cidaroida), a basal group within Echinidea (Smith et al., 2006), is characterized by the formation of variable numbers of micromeres and a late-ingressing skeletogenic mesenchyme (Schroeder, 1981; Wray and McClay, 1988).

These observations suggest that the ancestral echinoderm lacked micromeres, early-ingressing mesenchyme and an embryonic skeleton (Fig. 12, top row), but had NSM and a program of adult skeletogenesis. Early in echinoid evolution, the adult skeletogenic GRN, which probably included alx1 and many of the known biominalization genes, was co-opted by a sub-population of NSM cells, thereby creating an embryonic skeletogenic mesenchyme (Fig. 12, middle row). At this time, the formation of embryonic skeletogenic mesenchyme was not linked to micromere formation. Subsequently, in the lineage that gave rise to echinoids, the skeletogenic program again shifted earlier in development (Fig. 12, bottom row). At this time, activation of the GRN became tightly coupled to a strict system of unequal cleavage in the vegetal hemisphere (micromere formation). At the molecular level, new linkages (including pmar1) coupled the existing skeletogenic GRN to an ancient, maternally based system of embryo patterning (the β-catenin system). According to this scenario, the micromere-PMC GRN was established by forging new connections between these two pre-existing molecular programs.

We speculate that as this system evolved, a mechanism also arose for suppressing the skeletogenic potential of non-micromere-derived cells, thereby restricting skeletogenic differentiation to the early-ingressing mesenchyme (Fig. 12, bottom row, white arrows). The existence of this suppressive interaction is apparently ancient and widespread within the euechinoid species, as it has been observed in every species that has been carefully examined (at least seven species to date). According to this hypothesis, then, the regulative transfecting of NSM cells is a vestige of an ancient program of skeletogenesis. If true, then the regulation of the skeletogenic GRN in transfecting NSM cells may more closely resemble the pathway that operates in the late-ingressing, skeletogenic mesenchyme of cidaroid urchins than the micromere-based system seen in modern euechinoids.

Plasticity of early patterning in other species
The co-existence of early molecular asymmetries and developmental biases on the one hand, and plasticity/regulative potential on the other, is not unique to sea urchin development. Indeed, it seems likely that this is a universal feature of metazoan embryos. All animal embryos that have been well-studied exhibit characteristics of both mosaic and regulative development, and it has been argued that these terms have lost their utility (Lawrence and Levine, 2006). Recent work on early mouse development has provided strong evidence of developmental biases and molecular specialization at the earliest stages of cleavage (Zernicka-Goetz, 2006). These findings have sparked controversy, in part, because of the long history of work documenting the extensive regulative properties of mouse embryos. They are completely consistent, however, with the current picture of early sea urchin development. As the molecular and genetic mechanisms of early embryo patterning are elucidated we will gain a better understanding of how these processes respond to perturbations and underlie regulative phenomena.

We thank Kirsten Guss and Angela Bozak for their contributions to this work, and Simon Wu and David McClay for providing a full-length Lvpmar1 cDNA clone. This work was supported by NSF Grant IOB-0517214 (to C.A.E.).


