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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; Hörstadius, 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 and Angerer, 2003) and recent work suggests that the oral-aboral (OA) axis may also be entrained during oogenesis (Coffman et al., 2004). Thus, in sea urchins as in many other metazoans, the polarity of the unfertilized egg serves as a scaffold upon which the pattern of the early embryo is elaborated.

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The partitioning of the sea urchin embryo into distinct territories of gene expression begins during cleavage. Transcription commences immediately after fertilization and reaches its maximum rate after only four to five cleavage divisions (Davidson, 1986). The first territories of differential gene expression appear by the 16-cell stage (Di Bernardo et al., 1995; Nasir et al., 1995; Oliveri et al., 2002; Wang et al., 1996; Wikrmanayake et al., 2004). Other manifestations of molecular asymmetry are also evident at this stage, including the polarized nuclear accumulation of β-catenin and SoxB1 (Angerer et al., 2005; Weitzel et al., 2004). These findings raise an intriguing question: how can maternal polarity, early developmental biases, and the appearance of distinct territories of gene expression during cleavage be reconciled with the plasticity of cell fates that is observed during development?

Recent work concerning the early patterning of the sea urchin embryo has focused on transcriptional gene regulatory networks (GRNs) (Davidson et al., 2002; Oliveri and Davidson, 2004). One of the best-studied of these is the micromere-primary mesenchyme cell (PMC) GRN, which underlies the development of the embryonic skeleton. An early input into the micromere-PMC GRN is β-catenin, a maternally supplied protein which is stabilized preferentially in micromeres, most likely through the polarized localization of maternal regulators of canonical Wnt signaling (Logan et al., 1999; Weitzel et al., 2004; Ettensohn, 2006). β-catenin 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 (Ettensohn 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 ingression 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 ingression, 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 Ettensohn, 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 (Ettensohn and McClay, 1988; Ettensohn, 1990; Ettensohn, 1992). Surgical removal of both PMCs and NSM cells at the archenteron tip leads to fate-switching by presumptive endoderm cells (McClay and Logan, 1996). These studies indicate that many cells in the vegetal region retain the capacity to activate the skeletogenic program long after the onset of zygotic transcription and many hours after distinct GRNs have been activated in various vegetal territories of the embryo.

In this study, we have taken advantage of information that has emerged concerning the architecture of the micromere-PMC GRN to begin to dissect the molecular basis of plasticity in the early embryo. Our studies reveal that many features of the micromere-PMC GRN are faithfully recapitulated in transfating NSM cells. We provide evidence, however, that different inputs activate this GRN during normal and regulative development. We demonstrate that NSM cells, which appear to deploy a basal mesodermal GRN that shares several common elements with the PCM GRN, lack only an *alx1*-regulated sub-program for expression of a PMC fate. One hypothesis is that the regulative deployment of the PMC GRN in NSM cells is a vestige of an ancestral program of embryonic skeletogenesis.

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 Ettensohn, 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 Ettensohn and McClay (Ettensohn 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 but 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.

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 Ettensohn (Cheers and Ettensohn, 2004). LvAlx1 MO was described previously (Ettensohn 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, M_r=10,000; Invitrogen) into macromeres was carried out according to the method of Ruffins and Ettensohn (Ruffins and Ettensohn, 1996).

U0126 experiments

U0126 (Calbiochem) was prepared as a 5 mM stock in DMSO and stored at 4°C. Working dilutions (6-25 μM) were prepared in SW just before use. Embryos were placed in U0126 at the 2-cell stage or following PMC removal at the mesenchyme blastula stage, and were raised continuously in the dark in the presence of the drug. In control experiments, embryos were allowed to develop in equivalent concentrations of DMSO.

RT-PCR

RT-PCR using samples of five to ten embryos was carried out according to the method of Cheers and Ettensohn (Cheers and Ettensohn, 2005). PCR primers for detection of *Lvpmar1* mRNA were: 5′-TGTTCAACGA-CAACCAGTATCCTG-3′ (forward primer) and 5′-CACGACGCCC-AACTTCTTTG-3′ (reverse primer).

RESULTS

Activation of the micromere-PMC GRN during NSM transfating

Transfated 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 transfating and (3) the formation of an endoskeleton. We previously used a monoclonal antibody to show that transfated cells express PMC-specific proteins of the MSP130 family (Ettensohn and McClay, 1988).

To show directly that other downstream, skeletogenic genes in the PMC GRN are activated during transfating, we analyzed the expression of *Lvsm30*, *Lvsm50*, *Lvp16* and *Lvp58α* following PMC removal. LvSM30 and LvSM50 are secreted proteins occluded within the calcified spicule (Wilt and Ettensohn, 2007). LvP16 and LvP58α are novel, PMC-specific transmembrane proteins (Illies et al., 2002) (C.A.E., unpublished observations). LvP16 plays an essential role in skeletal rod elongation (Cheers and Ettensohn, 2005). LvP58α 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.

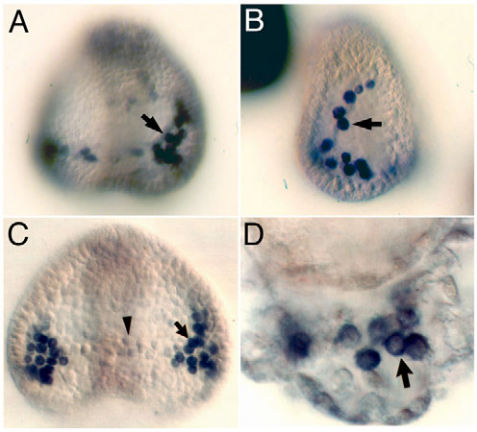

Fig. 1. WMISH analysis of skeletogenic genes during NSM transfating. (**A**) *Lvp16* (ventral view). (**B**) *Lvp58α* (lateral view). (**C**) *Lvsm30* (ventral view). (**D**) *Lvsm50* (scheitel region). Embryos were fixed 15 hours (A-C) or 36 hours (D) after PMC removal. Arrows point to the syncytial network of transfated cells. Arrowhead in C indicates transfated cells in the ventral region that do not express Lvsm30.

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 PMC chain (Fig. 1C). *Lvsm50* was expressed at high levels in the scheitel region at the pluteus stage (Fig. 1D).

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* (Ettensohn 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 (Ettensohn 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 cells types (data not shown). Because these experiments showed that NSM cells normally express *Lvets1/2* during gastrulation, we focused on the possible activation of the two other 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).

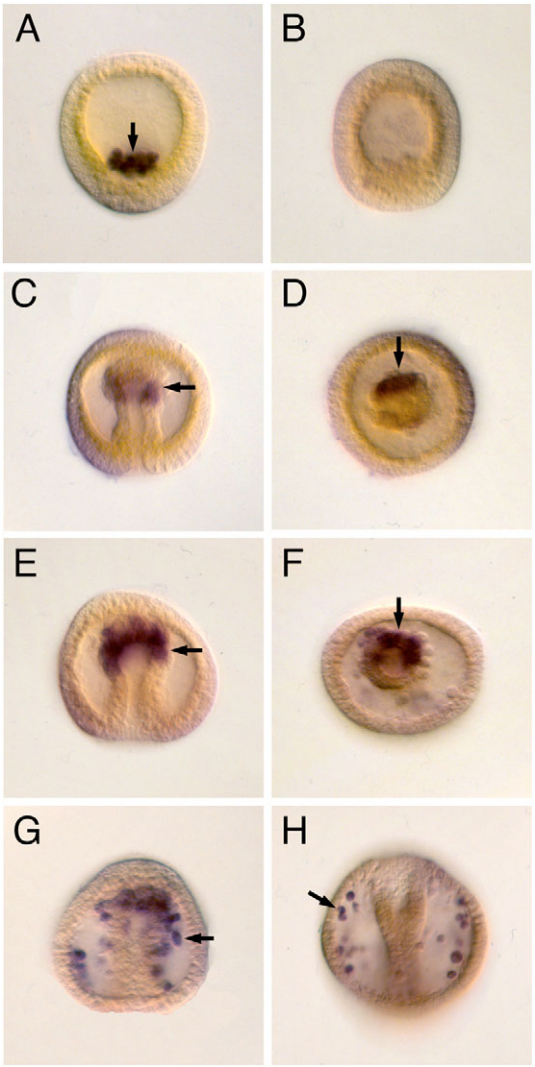

Fig. 2. PMC removal triggers ectopic expression of *Lvalx1* in cells near the tip of the archenteron. WMISH analysis of *Lvalx1* (A-G) and *Lvtbr* (H) expression. (**A**) Control mesenchyme blastula. *Lvalx1* is expressed specifically by PMCs (arrow). (**B**) A PMC-deficient embryo immediately after surgery. (**C**,**D**) PMC-deficient embryos 4 hours after surgery, viewed laterally (C) and along the AV axis (D). Cells near the tip of the archenteron express *Lvalx1* (C, arrow). In many embryos, *Lvalx1*-expressing cells are located predominantly on one side of the archenteron (D, arrow). (**E**,**F**) PMC-deficient embryos 5-6 hours after surgery, viewed laterally (E) and along the AV axis (F). *Lvalx1* is expressed by cells at the tip of the archenteron (arrows), some of which have begun to migrate out of the epithelium. (**G**) PMC-deficient embryo, 9-10 hours after surgery. *Lvalx1*-expressing cells are migrating within the blastocoel (e.g. arrow) and some remain associated with the archenteron tip. (**H**) *Lvtbr* expression in a PMC-deficient embryo, 10-11 hours after surgery. Transfating NSM cells are organizing in a subequatorial ring pattern and express *Lvtbr* (arrow).

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 ingression 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 LvAlx 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

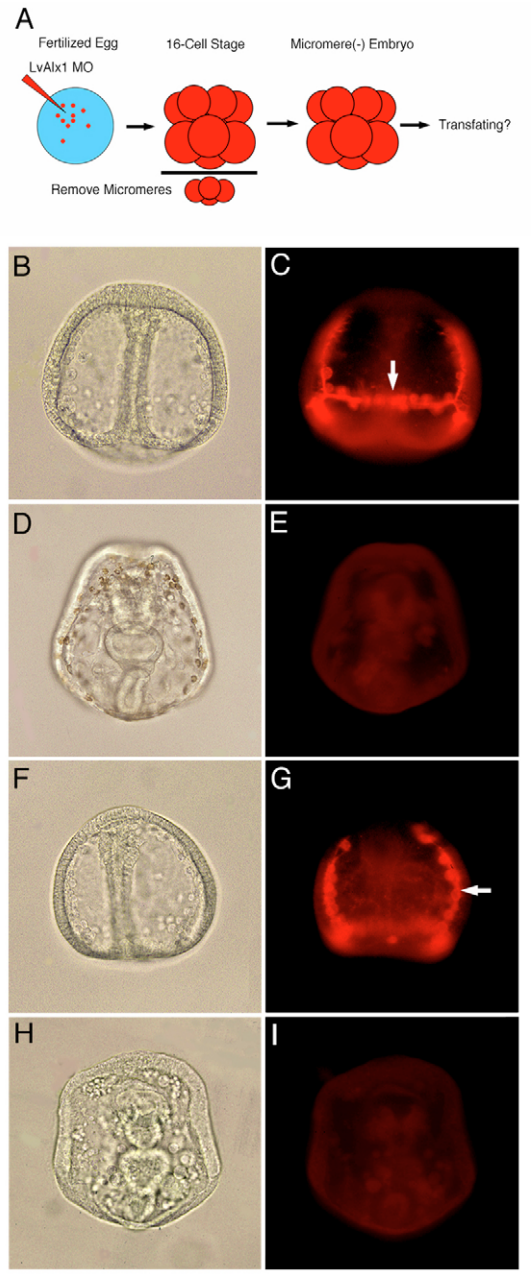

Fig. 3. LvAlx1 is required in macromere descendants for transfating. (**A**) Experimental protocol. (**B-I**) Embryos were immunostained with mAb 6a9 and the same embryos were photographed using both brightfield (left column) and epifluorescence (right column) optics. (B,C) Control late gastrula stage embryo, 20 hours postfertilization. (D,E) LvAlx1 MO-injected embryo, 48 hours postfertilization. Most morphogenetic processes are unaffected by LvAlx1 knockdown but the embryo lacks 6a9-positive cells and skeletal elements. (F,G) Micromere-deficient [micromere(–)] embryo, 24 hours postfertilization. Transfating has occurred, leading to the formation of many 6a9-positive cells (arrow) and two skeletal primordia. (H,I) LvAlx1 MO-injected, micromere-deficient embryo, 48 postfertilization. The embryo has undergone extensive morphogenetic changes but lacks 6a9-positive cells and a skeleton.

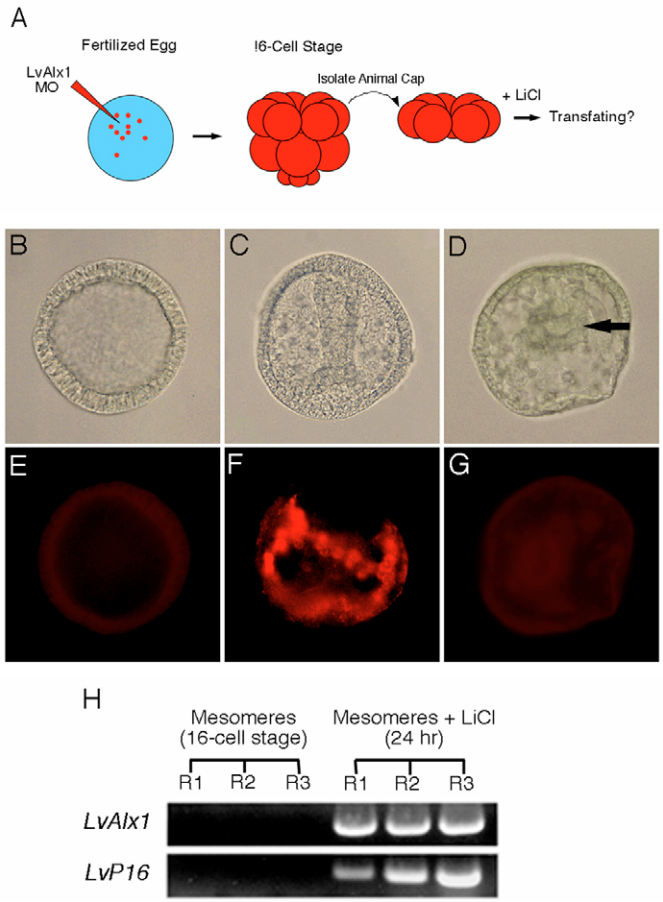

Fig. 4. LvAlx1 is required in mesomere descendants for transfating. (**A**) Experimental protocol. (**B-G**) Embryos were immunostained with mAb 6a9 and the same embryos were photographed using brightfield (B-D) and epifluorescence (E-G) optics. (B,E) Animal cap isolated at the 16-cell stage and cultured for 24 hours in the absence of LiCl. The embryo lacks mesoderm and endoderm and no 6a9-positive cells are present. (C,F) Animal cap treated with 50 mM LiCl for 3 hours after the operation and cultured for 24 hours. Endoderm and mesoderm have formed, including many 6a9-positive cells. (D,G) Animal cap isolated from a LvAlx1 MO-injected embryo, treated with 50 mM LiCl, and cultured for 48 hours. Although a gut (arrow) has formed, no 6a9-positive cells are present. (**H**) RT-PCR experiments showing that animal caps treated with 50 mM LiCl express *Lvalx1* and one of its downstream targets (*Lvp16*). Each experiment was repeated three times (R1-R3).

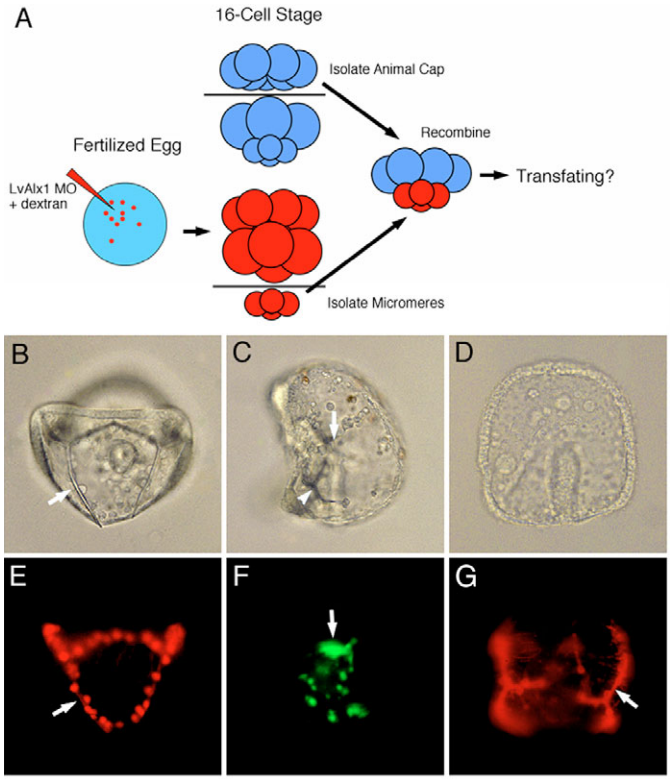

Fig. 5. *Lvalx1* is upstream of the signal that suppresses NSM transfating. (**A**) Experimental protocol. (**B-G**) Recombinant embryos were photographed using brightfield (upper row) and epifluorescence (lower row) optics. (B,E) Animal cap + micromere recombinant, 36 hours after fertilization. Micromere progeny (labeled with Rhodamine dextran) have formed the embryonic skeleton (arrow) and have induced the formation of a complete embryonic axis. (C,F) Animal cap + LvAlx1 MO-micromere recombinant, 48 hours after fertilization. LvAlx1 MO-injected micromeres have induced a complete embryonic axis. In contrast to B,E, however, the progeny of the micromeres (labeled with fluorescein dextran) are not arranged along the skeletal rods (C, arrowhead). Instead, they are scattered in the blastocoel or remain associated with the tip of the archenteron (arrow). (D,G) Animal cap + LvAlx1 MO-micromere recombinant, 48 hours after fertilization. Mesomere descendants have transfated, as shown by 6a9 immunostaining, and are associated with skeletal elements (G, arrow).

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

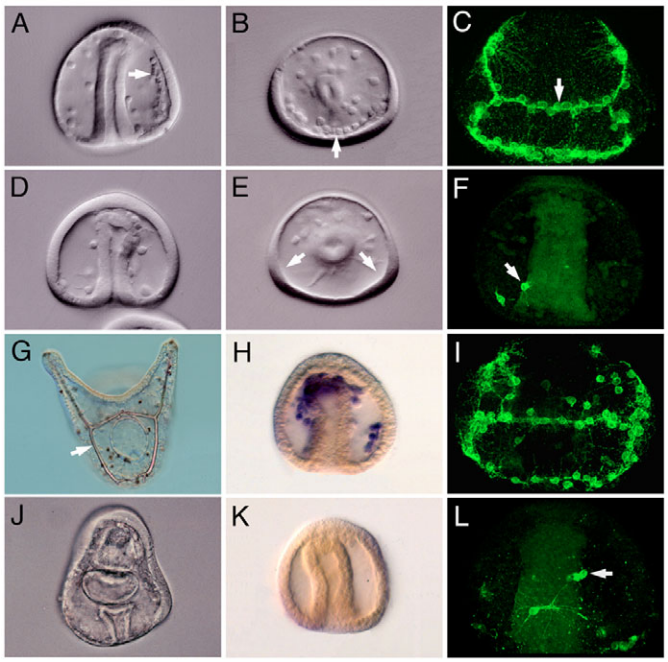

Fig. 6. MAPK signaling is required for transfating. Right panels show embryo whole mounts immunostained with mAb 6a9 and examined by confocal microscopy. The remaining panels show living embryos viewed with differential interference optics, except H and K, which are in situ hybridizations. (**A-C**) Control embryos at the late gastrula stage. 65-70 PMCs are arranged in a subequatorial ring pattern (arrow). (**D-F**) Embryos treated with 6 μM U0126 from the 2-cell stage. Arrows in E indicate locations adjacent to ectodermal thickenings where ventrolateral clusters of PMCs normally form. Arrow in F indicates one of two 6a9-positive cells that formed in this embryo, which was treated with U0126 until sibling controls reached the prism stage. (**G-I**) Control PMC-deficient embryos (not treated with U0126). Transfating is indicated by skeleton formation (G; 24 hours after surgery), *Lvalx1* mRNA expression (H; 8 hours after surgery), and 6a9 immunostaining (I; 12 hours after surgery). (**J-L**) PMC-deficient embryos transferred to 6 μM U0126 immediately after surgery (J,K and L show embryos 24, 8 and 24 hours after surgery, respectively).

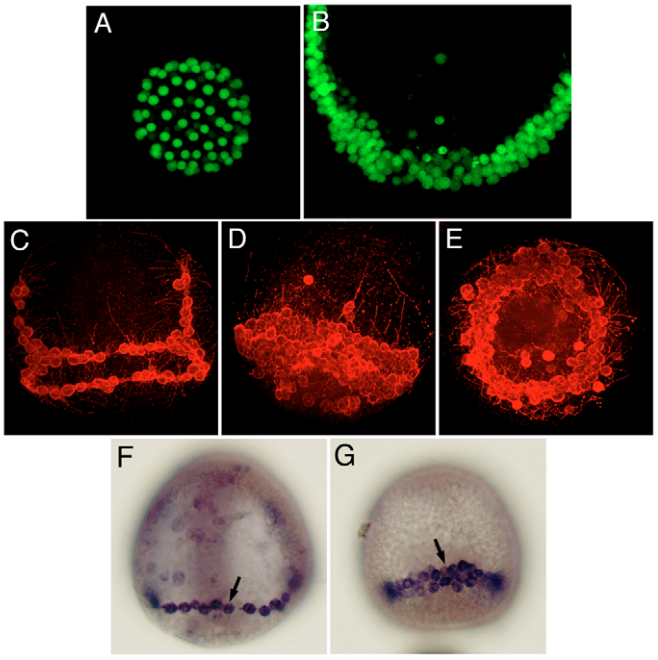

Fig. 7. Overexpression of LvAlx1 is sufficient to increase numbers of skeletogenic cells. (**A**) Projection of a z-stack of confocal images of a 6 hour (early blastula) embryo expressing LvAlx1-GFP. LvAlx1 protein accumulates in the nuclei of all blastomeres. (**B**) Projection of a z-stack of the vegetal plate of a mesenchyme blastula-stage embryo expressing LvAlx1-GFP. Cells throughout the vegetal plate have tagged protein in their nuclei. (**C-E**) Embryos expressing wild-type or mutant forms of LvAlx1, fixed at the late gastrula stage and immunostained with mAb 6a9. (C) Embryo injected with *Lvalx1.STOP* mRNA (0.75 mg/ml). Numbers of 6a9-positive cells are similar to those observed in uninjected sibling embryos (Fig. 6A and Fig. 8). (D,E) Lateral and vegetal views of two different embryos injected with *Lvalx1.WT* mRNA (0.38 mg/ml). These embryos have large numbers of 6a9-positive cells (100-120 cells) arranged in a radially symmetrical band. (**F**) *Lvp16* expression (arrow) in PMCs of a control embryo. (**G**) A sibling embryo injected with *Lvalx1.WT* mRNA (0.38 mg/ml). Overexpression of LvAlx1 leads to increased numbers of *Lvp16*-expressing cells, which are arranged in a circumferential belt (arrow).

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 (untagged) 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 ring between the equator and the vegetal pole. These cells also expressed *Lvp16* (Fig. 7F,G). Injection of higher concentrations of *Lvalx1* mRNA (1.5 mg/ml or higher)

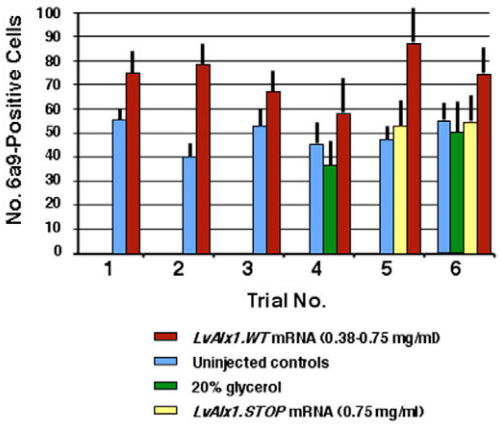

Fig. 8. The effect of Alx1 overexpression on numbers of 6a9-positive cells. The results of six independent trials are shown. Each vertical bar indicates a mean calculated from 10-30 embryos. Vertical lines represent one standard deviation. 20% glycerol, the mRNA carrier solution, was used as a control; *LvAlx1.STOP* is a mutant that cannot bind to DNA.

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).

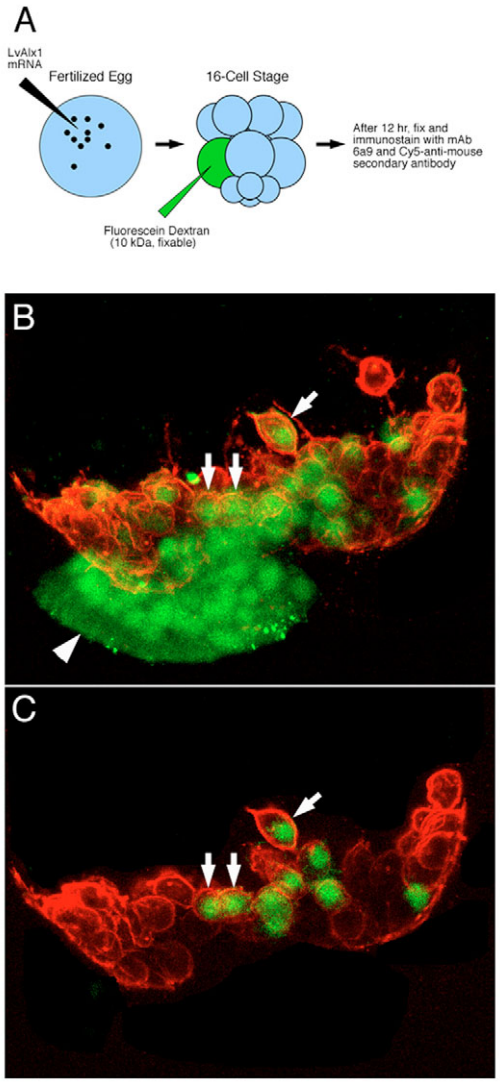

Fig. 9. Overexpression of LvAlx1 activates the PMC GRN in macromere descendants. (**A**) Experimental protocol. Fertilized eggs were injected with *Lvalx1.WT* mRNA. At the 16-cell stage, one macromere was injected with fluorescein dextran. After 12 hours, the embryos were fixed, immunostained with mAb 6a9 and a Cy5-conjugated secondary antibody, and examined by confocal microscopy. (**B**) A 2-D projection of a complete z-stack (16 images) of an experimental embryo. Fluorescent dextran is shown in green and 6a9 immunostaining in red. The macromere injected with dextran has contributed to a patch of cells outside the blastopore (arrowhead) as well as cells in the archenteron and a few migrating mesenchyme cells (arrows). This low M_r dextran is concentrated in cell nuclei (Hodor and Ettensohn, 1998). (**C**) A 2-D projection of a partial z-stack (4 images) from the same embryo. This projection shows more clearly that some 6a9-positive cells are also labeled with fluorescent dextran (arrows).

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

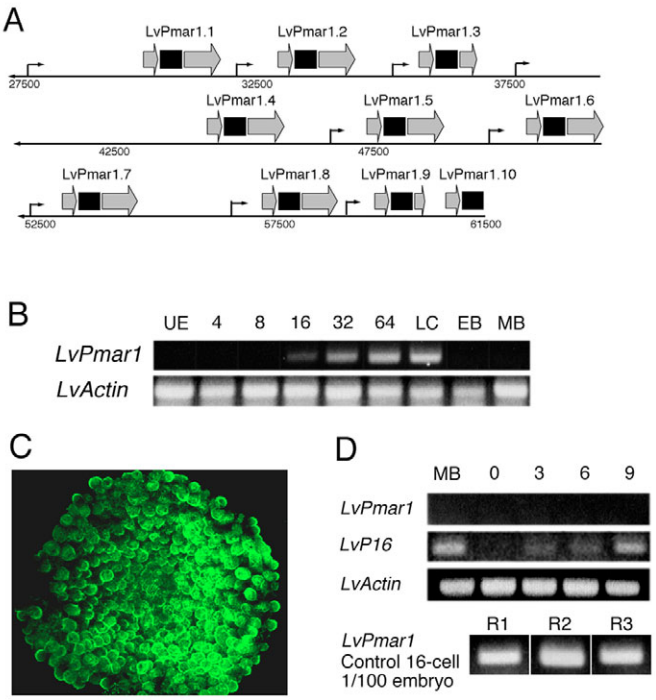

Fig. 10. *Lvpmar1* does not activate the skeletogenic GRN network during NSM transfating. (**A**) The *Lvpmar1* locus. (**B**) Developmental RT-PCR analysis of *Lvpmar1* expression. Stages shown are unfertilized egg (UE), 4-, 8-, 16-, 32- and 64-cell stages, late cleavage (LC), early blastula (EB) and mesenchyme blastula (MB). (**C**) Overexpression of *Lvpmar1* activates the PMC GRN in all cells of the embryo. *Lvpmar1* mRNA (100 μg/ml) was injected into fertilized eggs and after 24 hours the embryos were fixed and immunostained with mAb 6a9. *Lvpmar1* causes a transformation of all cells to a mesenchymal, 6a9-positive phenotype. (**D**) RT-PCR analysis of *Lvpmar1*, *Lvp16* and *Lvactin* mRNA expression in PMC-deficient embryos. MB, control mesenchyme blastula stage embryos. Other lanes show PMC-deficient embryos, collected 0, 3, 6 and 9 hours after PMC depletion. *Lvp16*, a target of *Lvpmar1* and *Lvalx1*, is expressed within 3 hours after PMC depletion and at higher levels at 9 hours. *Lvpmar1* mRNA is not detectable at any of the stages examined (the amount of starting material at each stage was equivalent to 1 embryo). Bottom panels show control experiments using identical RT-PCR conditions but with cell lysates prepared from 16-cell stage embryos. *Lvpmar1* can be detected when the amount of starting material is equivalent to just 1/100 embryo. R1-R3 are three independent replicates of the experiment, performed using three different batches of embryos.

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 (Langelan 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 Ettensohn 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; Okazaki, 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.,

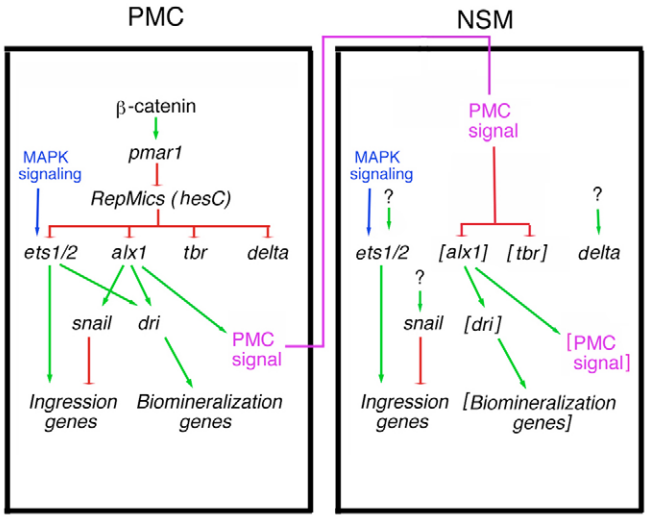

Fig. 11. A comparison of GRN architecture in the large micromere-PMC lineage and NSM cells. In presumptive PMCs (left), maternal β-catenin activates *pmar1*, which represses *hesC*. Components of the MAPK signaling pathway are activated in the PMC lineage by an unknown mechanism and bring about the phosphorylation and activation of Ets1/2. *Ets1/2* and *alx1* regulate genes that control ingression, migration, and biomineralization, and *alx1* has an essential input into PMC signaling. Note that although *snail* is a transcriptional repressor, it stimulates ingression (Wu and McClay, 2007). In presumptive NSM cells (right), several components of the PMC GRN (e.g. *ets1/2*, *delta* and *snail*) are normally expressed, although little is known concerning their upstream regulators. MAPK signaling is active and presumably causes the phosphorylation of Ets1/2. *Alx1* and *tbr* are normally repressed, directly or indirectly, by the PMC-derived signal (genes not normally expressed in NSM cells are shown in brackets). Key downstream targets of *alx1* in the skeletogenic pathway are therefore not expressed. Elimination of the signal induces expression of *alx1*, which is sufficient to engage all essential, missing elements of the PMC GRN. One consequence of the activation of the PMC GRN in NSM cells is the acquisition of PMC-specific signaling properties (Ettensohn and Ruffins, 1993).

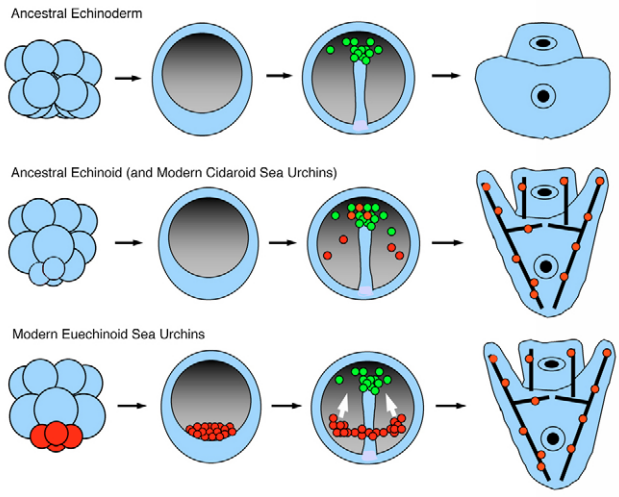

Fig. 12. Possible evolutionary changes in the deployment of the skeletogenic GRN. NSM shown as green cells; micromeres and skeletogenic mesenchyme cells are shown in red. See Discussion for details.

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 transfating. 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 transfating 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 transfating, 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 et al., 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 transfating 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 (Oliveri et al., 2002; Oliveri 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 Cidaroidea), a basal group within Echinoidea (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 biomineralization 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 euechinoids, 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 urchins, 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 transfating of NSM cells is a vestige of an ancient program of skeletogenesis. If true, then the regulation of the skeletogenic GRN in transfating 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.

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