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Regulative deployment of the skeletogenic gene regulatory network during sea urchin development

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SUMMARY

The well-known regulative properties of the sea urchin embryo, coupled with the recent elucidation of gene regulatory networks (GRNs) that underlie cell specification, make this a valuable experimental model for analyzing developmental plasticity. In the sea urchin, the primary mesenchyme cell (PMC) GRN controls the development of the embryonic skeleton. Remarkably, experimental manipulations reveal that this GRN can be activated in almost any cell of the embryo. Here, we focus on the activation of the PMC GRN during gastrulation by non-skeletogenic mesoderm (NSM) cells and by endoderm cells. We show that most transfating NSM cells are prospective blastocoelar cells, not prospective pigment cells, as was previously believed. Earlier work showed that the regulative deployment of the GRN, unlike its deployment in the micromere-PMC lineage, is independent of the transcriptional repressor Pmar1. In this work, we identify several additional differences in the upstream regulation of the GRN during normal and regulative development. We provide evidence that, despite these changes in the upstream regulation of the network, downstream regulatory genes and key morphoregulatory genes are deployed in transfating NSM cells in a fashion that recapitulates the normal deployment of the GRN, and which can account for the striking changes in migratory behavior that accompany NSM transfating. Finally, we report that mitotic cell division is not required for genomic reprogramming in this system, either within a germ layer (NSM transfating) or across a germ layer boundary (endoderm transfating).

KEY WORDS: Gene regulatory networks, Regulative development, Sea urchin embryo

INTRODUCTION

A fundamental question in development concerns the molecular mechanisms that underlie cellular plasticity. The plasticity of embryonic cells has been documented in almost all metazoan embryos that are used for developmental studies, challenging the view that cell fates are rigidly and immutably fixed. More recently, the finding that somatic cells can be reprogrammed to generate embryonic pluripotent stem cells, with potential uses in regenerative medicine, has led to an increased interest in understanding the process of cellular reprogramming. The sea urchin embryo is a valuable experimental model to study questions related to developmental plasticity because of its extensive and well-described regulative properties. In addition, in recent years, a systems biology approach has been used to generate detailed transcriptional GRNs for the different cell lineages of this embryo. This presents a unique opportunity to approach questions related to developmental plasticity in terms of the epigenetic regulation of GRNs.

During normal development, the skeletogenic cells are the descendants of the four large micromeres, cells that arise from unequal fourth and fifth cleavage divisions at the vegetal pole of the embryo. At the blastula stage, the descendants of the large micromeres occupy the central region of the vegetal plate and are surrounded by NSM cells. At the start of gastrulation, the large micromere descendants undergo an epithelial-mesenchymal transition (EMT) and migrate into the blastocoel; these cells are referred to thereafter as PMCs. The PMCs migrate to specific positions in the blastocoel and secrete the calcified endoskeleton of the larva. Later in gastrulation, two populations of NSM cells also undergo EMT; first pigment cells, and later a population of fibroblast-like cells known as blastocoelar cells (Gibson and Burke, 1985; Tamboline and Burke, 1992). Other NSM cells give rise to circumesophageal muscle cells and the cells of the coelomic pouches (Ruffins and Ettensohn, 1996).

The PMC GRN is currently one of the best-understood developmental GRNs (Oliveri et al., 2008; Ettensohn, 2009). The activation of this GRN is dependent on the stabilization of β-catenin in the vegetal region of the embryo (Wikramanayake et al., 1998; Logan et al., 1999; Ettensohn, 2006). A direct target of β-catenin is the transcriptional repressor *pmar1* (Kitamura et al., 2002; Oliveri et al., 2002). By a de-repression mechanism, Pmar1 is believed to activate the signaling gene *delta* (Oliveri et al., 2002; Sweet et al., 2002), and a suite of early regulatory genes, which includes *alx1* (Ettensohn et al., 2003), *tbrain* (Fuchikami et al., 2002; Oliveri et al., 2002), *ets1* (Kurokawa et al., 1999), specifically in the large micromere territory. These transcription factors activate other regulatory genes and, ultimately, genes that control PMC morphogenesis and biomineralization. The mitogen-activated protein kinase (MAPK) signaling pathway is required for PMC specification and ingression; this pathway plays a role in maintaining the expression of the key transcription factor *alx1* and other genes through the phosphorylation of Ets1 (Röttinger et al., 2004; Sharma and Ettensohn, 2010). The large micromeres also play an essential role in the induction of the NSM; elimination of Delta or Notch function results in embryos that lack pigment cells and have reduced numbers of blastocoelar and muscle cells (Sherwood and McClay, 1999; Sweet et al., 2002).

Surgical removal of PMCs at the mesenchyme blastula stage results in activation of the skeletogenic GRN by NSM cells, a process referred to as NSM transfating (Fig. 1). NSM transfating is associated with the expression of *alx1* and several downstream biomineralization-related genes. Unlike normal development, the

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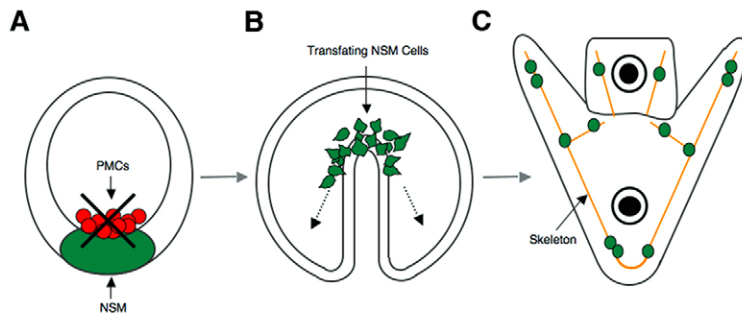

Fig. 1. Primary mesenchyme cell (PMC) depletion in sea urchin embryos induces non-skeletogenic mesoderm (NSM) cells to adopt a skeletogenic fate. (**A-C**) Surgical removal of PMCs at the mesenchyme blastula stage (A) induces a subpopulation of NSM cells to activate the skeletogenic gene regulatory network (GRN). These cells migrate away from the tip of the archenteron at the late gastrula stage (B) and later secrete a normally patterned skeleton (C). Dotted arrows indicate the migration of transfated NSM cells to PMC-specific target sites.

activation of *alx1* by transfating NSM cells has been shown to occur by a Pmar1-independent mechanism, a finding that points to the presence of novel upstream inputs into the network during regulative development (Ettensohn et al., 2007). In addition, the same study showed that regulative development requires active MAPK signaling for the synthesis of the larval skeleton, although the role of MAPK in the regulation of the skeletogenic GRN was not explored further. Other surgical manipulations result in the ectopic activation of the PMC GRN by other cell types; for example, the removal of both the PMCs and the NSM results in the activation of this network by presumptive endoderm cells (McClay and Logan, 1996).

The purpose of this study was to dissect further the molecular basis of developmental plasticity in the sea urchin embryo by analyzing the regulative deployment of the skeletogenic GRN. Our findings modify the current view of the population of NSM cells that transfates and, therefore, the nature and extent of the genomic reprogramming that occurs. We identify several differences in the upstream activation of the GRN in transfating cells compared with the large micromere-PMC lineage, but find that the faithful recapitulation of intermediate regulatory layers of the network and the activation of key morphoregulatory genes mediate the striking changes in cell behavior that are associated with transfating. To compare the mechanisms that activate the skeletogenic GRN in different embryonic lineages, we extend this approach to the deployment of the GRN by endoderm cells and provide evidence that this occurs by the re-specification of an NSM territory. Finally, we show that mitotic cell division is not required for the re-programming of NSM or endoderm cells to a skeletogenic phenotype.

MATERIALS AND METHODS

Animals

Adult *Lytechinus variegatus* were obtained from Reeftopia (Key West, FL, USA). Gametes were obtained by intracoelomic injection of 0.5 M KCl and embryos were cultured at 23°C.

Fluorescent whole-mount in situ hybridization (F-WMISH)

Single and two-color F-WMISH were performed as described previously (Sharma and Ettensohn, 2010). Cell nuclei were stained by incubating embryos in 0.5 μg/ml Hoechst 33342 in PBST (0.1% Tween-20 in phosphate-buffered saline) for 5 minutes, followed by several rinses in PBST.

Microscopy and image processing

z-stacks were collected at 1 μm intervals using a Zeiss LSM 510 meta/UV DuoScan spectral confocal microscope and a 40× oil immersion lens. Each image shown in the figures is a two-dimensional projection of 10-20 digital sections obtained using the average intensity projection tool of ImageJ.

Morpholino microinjections

Lvdelta morpholino (MO) (Sweet et al., 2002) was obtained from Gene Tools, LLC (Philomath, OR, USA). The injection solution consisted of 2 mM MO in 20% glycerol.

U0126 treatment

PMC(–) or PMC(–), arch(–) embryos were treated with U0126 at a concentration of 6 μM immediately after surgery until the desired developmental stage, when the embryos were fixed for F-WMISH analysis.

Microsurgery

PMC removal and archenteron removal were carried out at the mesenchyme blastula and early gastrula stages, respectively, as described previously (Ettensohn and McClay, 1988; McClay and Logan, 1996).

Cell proliferation assay

EdU (5-ethynyl-2′-deoxyuridine) labeling and detection by click chemistry were carried out using the Click-iT EdU Cell Proliferation Kit (Invitrogen). Control experiments showed that EdU remained stable in seawater for at least 24 hours and was incorporated within 15 minutes by cells that were in S phase. Aphidicolin, an inhibitor of DNA polymerase I, effectively blocks DNA replication and cell division in the sea urchin embryo (Stephens et al., 1986). Control studies confirmed that addition of 0.3 μg/ml aphidicolin to the medium blocked the incorporation of EdU within 15 minutes. For PMC-removal experiments, embryos were placed in 1 μM EdU 30-60 minutes prior to PMC removal and were incubated continuously in the presence of EdU. Seven to eleven hours after PMC removal, embryos were fixed and stained with monoclonal antibody (MAb) 6a9 (Ettensohn and McClay, 1988). For PMC and archenteron removal experiments, PMCs were removed at the mesenchyme blastula stage and the archenteron was removed 2-3 hours later, at the early gastrula stage. Embryos were transferred to 1 μM EdU 1 hour prior to the removal of the archenteron and were incubated continuously in the presence of the label. Embryos were fixed and immunostained with MAb 6a9 12 or 22 hours after removal of the archenteron. For all experiments that used aphidicolin, the inhibitor was added to the seawater 30 minutes prior to the addition of EdU.

RESULTS

During transfating, the upstream regulation of the PMC GRN is modified but the downstream network is faithfully recapitulated

The transcription factor *alx1*, which in the micromere-PMC GRN is regulated by a de-repression system mediated by *pmar1*, is activated in NSM cells by novel, *pmar1*-independent input(s) (Ettensohn et al., 2007). To analyze the network in transfating cells in greater detail, we first focused on the activation of two other early genes in the network: *delta* and *tbr*. *delta* is activated zygotically and is expressed in the large micromeres at the early blastula stage. *delta* is subsequently downregulated in the micromeres and is expressed transiently in the NSM until the late mesenchyme blastula-early gastrula stage. The only known function of micromere-derived Delta is the induction of the NSM (Sweet et al., 2002), a function that is probably not required at the stage at which NSM transfating occurs. To test whether *delta* is activated during transfating, two-color F-WMISH was performed on PMC(–) embryos at 2 and 5 hours post-depletion (hpd). F-

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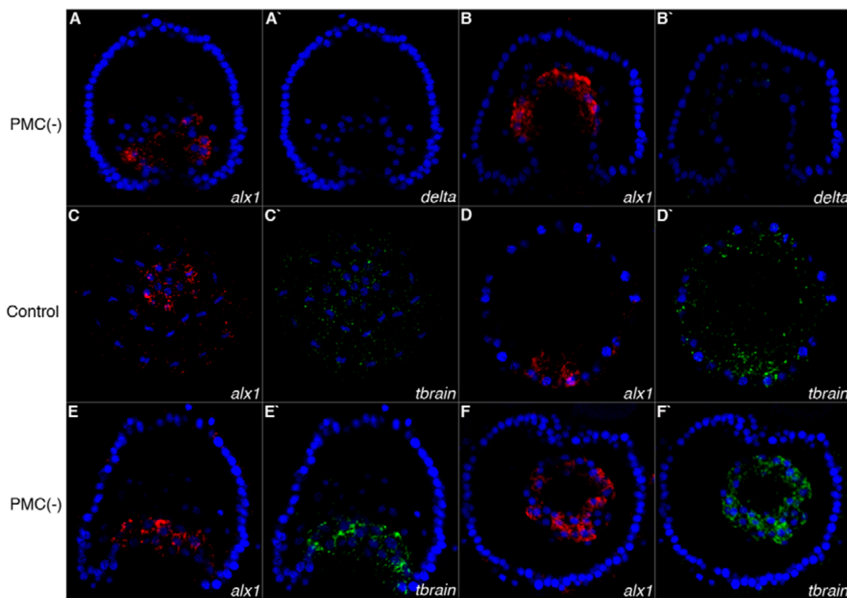

Fig. 2. The early skeletogenic gene regulatory network (GRN) gene *tbr*, but not *delta*, is activated by transfating non-skeletogenic mesoderm (NSM) cells. The expression of *tbr* and *delta* in PMC(–) sea urchin embryos was analyzed at 2 (A,A′,E,E′) and 5 (B,B′,F,F′) hpd using two-color fluorescent whole-mount in situ hybridization (F-WMISH) in combination with *alx1*. (**A-B′**) *delta* is not expressed by transfating cells (A′,B′), which are unambiguously identified by *alx1* expression (red, A,B). (**C-D′**) During normal development, *alx1* transcripts are detected at the early blastula stage (5 hpf) (C, vegetal view), whereas *tbr* transcripts are detected at the mid-blastula stage (D′), showing that *alx1* expression precedes the expression of *tbr*. (**E-F′**) *Tbr* (green) is activated in transfating cells as early as 2 hpd (E′), the earliest time at which *alx1* transcripts can also be detected (E). Embryos were counterstained with Hoechst dye (blue).

WMISH showed that *delta* was not activated in the transfating cells, whereas *alx1* expression was clearly detectable in the same embryos (Fig. 2A-B′).

During normal development, *tbr* mRNA is provided maternally and zygotic activation of *tbr* occurs only in the large micromere territory (Fuchikami et al., 2002). The enrichment of *tbr* transcripts in the large micromere descendants was first detected by F-WMISH at the mid-blastula stage, 6-7 hours post-fertilization (hpf) (Fig. 2D′) but *alx1* transcripts could be detected earlier, at the early blastula stage (5 hpf) (Fig. 2C). Therefore, during normal development, the accumulation of *alx1* transcripts precedes the zygotic activation of *tbr*. We found by F-WMISH that *tbr* was activated in PMC(–) embryos as early as 2-3 hpd, similar to the time when we first began to detect *alx1* transcripts, suggesting that both *alx1* and *tbr* were activated quite early, and nearly simultaneously, during transfating. To test more directly whether *alx1* and *tbr* were activated simultaneously in the transfating cells, we performed two-color F-WMISH with *alx1* and *tbr* probes on PMC(–) embryos at 2 and 3 hpd. At 2 hpd, when the process of transfating was just being initiated, we could detect the expression of *alx1* in only four out of 11 embryos, and every embryo that expressed *alx1* also expressed *tbr* (Fig. 2E,E′). By 3 hpd, we could detect the activation of both *alx1* and *tbr* in every embryo (n=10) (Fig. 2F,F′). Our findings indicate that these early regulatory genes are activated in a different temporal sequence during normal and regulative development, i.e. they are activated nearly simultaneously in transfating NSM cells, whereas the activation of *alx1* precedes that of *tbr* in the large micromere-PMC lineage.

We next examined the expression in PMC(–) embryos of several downstream genes in the skeletogenic GRN. We focused on the expression of five genes: *dri* (Amore et al., 2003), *foxB* (Minokawa et al., 2004), *jun* (Oliveri et al., 2008), *vegfr-Ig-10* (Duloquin et al., 2007) and *fgfr-2* (Röttinger et al., 2008). *dri*, *foxB* and *jun* are late regulatory genes; *dri* and *foxB* are downstream targets of *alx1* (Oliveri et al., 2008) but nothing is known about the upstream regulation of *jun*. *vegfr-Ig-10* and *fgfr-2* are tyrosine kinase receptors that have recently been implicated in PMC guidance and differentiation. The orthologs of these genes in *L. variegatus* were cloned using degenerate RT-PCR and RACE. We asked (1) whether these genes were activated during transfating, (2) whether they were co-expressed in precisely the same cells, and (3) whether their timing of activation mimicked that seen during normal development. To address these questions, we performed two-color F-WMISH for each gene in combination with *alx1* at different times after PMC removal. These studies showed that each of the five genes was activated in the same cells that expressed *alx1* during transfating (Fig. 3A-E″). This analysis also suggested that the order of activation of these genes was similar to that observed during normal development. For example, *fgfr-2* and *vegfr-Ig-10* are ordinarily activated later than the upstream regulatory genes in the network (Duloquin et al., 2007; Oliveri et al., 2008; Röttinger et al., 2008). In PMC(–) embryos, these genes were activated only when transfating NSM cells began to migrate away from the tip of the archenteron (10-11 hpd) (Fig. 3D-E″), whereas transfated cells that had still not acquired a mesenchymal character did not have detectable levels of these mRNAs (Fig. 3D-E″, arrowhead).

Presumptive blastocoelar cells transfate following PMC removal

NSM cells occupy the central region of the vegetal plate at the mesenchyme blastula stage, whereas during gastrulation these cells are located at the tip of the growing archenteron (Ruffins and Ettensohn, 1996). PMC removal induces the activation of *alx1* in cells at the tip of the archenteron (Ettensohn et al., 2007), indicating that the transfating cells lie within the NSM territory. It is unclear, however, whether all cells within this territory activate the network, or whether it is deployed by a specific subpopulation of NSM cells.

Pigment cells are the first NSM cells to undergo EMT. By the mid-gastrula stage, most pigment cells have migrated into the aboral ectoderm (Gibson and Burke, 1985). We confirmed this pattern of migration in *L. variegatus* by F-WMISH with *pks*, a gene specifically expressed by pigment cells (Calestani et al., 2003) (see Fig. S1 in the supplementary material). During transfating, *alx1* expression was detected within 2-3 hpd in cells that were located near the tip of the archenteron. These cells were epithelial in origin and maintained their epithelial character until the late gastrula stage (10-11 hpd), when they became mesenchymal and migrated away from the tip of the archenteron**.** Based on their very different locations in the embryo, it seemed unlikely that pigment cells

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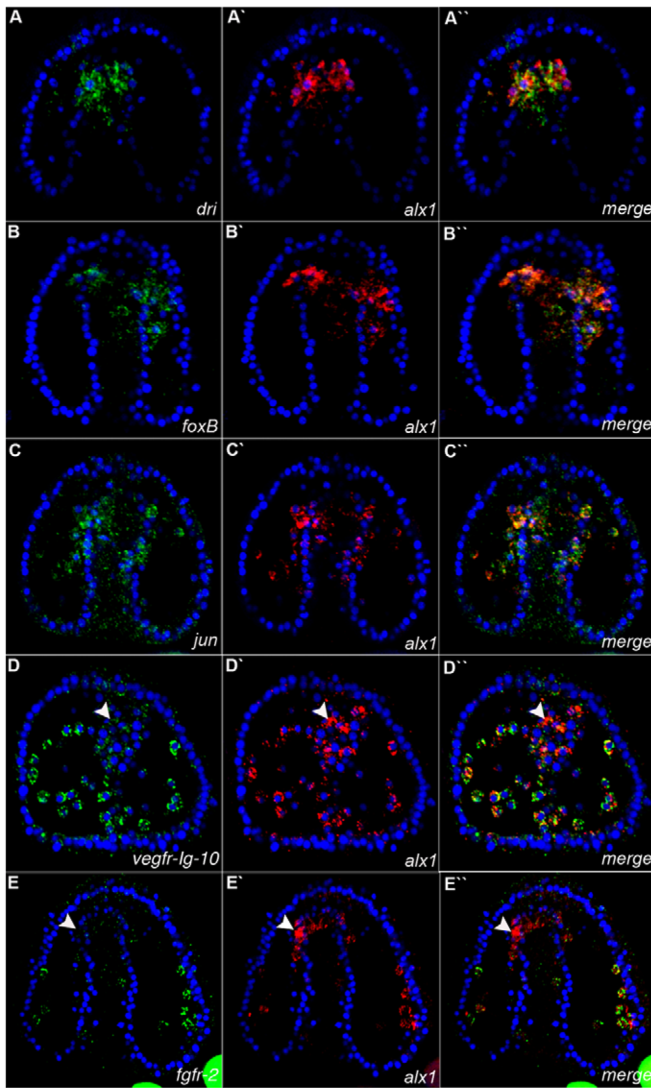
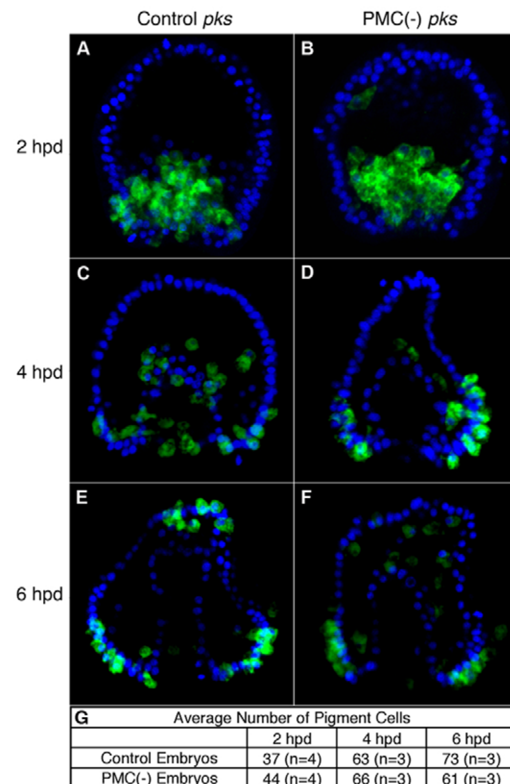


Fig. 3. The downstream primary mesenchyme cell gene regulatory network (PMC GRN) is faithfully recapitulated during non-skeletogenic mesoderm (NSM) transfating. (**A-E″**) The expression of *dri*, *foxB*, *jun*, *vegfr-Ig-10* and *fgfr* (green) was analyzed by two-color fluorescent whole-mount in situ hybridization (F-WMISH) in combination with *alx1* (red) in PMC(–) sea urchin embryos at 5-11 hpd. Each image is a projection of several confocal sections. The regulatory genes *dri*, *foxB* and *jun* are expressed when transfating NSM cells are still associated with the archenteron (A-C″). Expression of *vegfr-Ig-10* and *fgfr* is detectable only when the transfating cells begin to migrate away from the tip (D-E″); no expression is seen in cells that are still associated with the archenteron (arrowheads in D-E″). Embryos were counterstained with Hoechst dye (blue).



Average Number of Pigment Cells	2 hpd	4 hpd	6 hpd
Control Embryos	37 (n=4)	63 (n=3)	73 (n=3)
PMC(-) Embryos	44 (n=4)	66 (n=3)	61 (n=3)

Fig. 4. The specification and migration of pigment cells is unaffected by primary mesenchyme cell (PMC) removal. Pigment cell morphogenesis was compared in control and PMC(–) sea urchin embryos using *pks* as a marker. Each panel is a merged image of *z*-projections of confocal stacks (green, *pks*; blue, Hoechst). (**A**,**C**,**E**) Control embryos at 2 (A), 4 (C) and 6 (E) hpd showing the normal pattern of pigment cell migration. (**B**,**D**,**F**) Sibling PMC(–) embryos at 2 (B), 4 (D) and 6 (F) hpd. Removal of PMCs has no effect on the specification or migration of pigment cells. (**G**) Table showing comparable numbers of pigment cells in control and sibling PMC(–) embryos at 2, 4 and 6 hpd.

contributed to the population of *alx1*(+) cells. We considered the possibility, however, that microsurgical depletion of PMCs might alter the pattern of pigment cell migration. To test this possibility, we examined the specification and migration of pigment cells in PMC(–) embryos at 2, 4 and 6 hpd by F-WMISH with *pks*. We found that the number and pattern of migration of pigment cells in PMC(–) and control embryos were indistinguishable (Fig. 4A-G), confirming that pigment cells do not contribute significantly to the population of transfating NSM cells.

Inhibiting the Delta-Notch signaling pathway using a Delta MO, or misexpressing a dominant negative form of Notch, leads to the development of embryos that completely lack pigment cells (Sherwood and McClay, 1999; Sweet at al., 2002). We assayed the expression of the pigment cell marker *pks* and the blastocoelar cell marker *scl* in Delta MO-injected embryos. We found that the expression of *pks* was completely blocked in such embryos, but the expression of *scl* was still detectable (Fig. 5A-D). These observations confirmed that the Delta MO blocked pigment cell specification but had little effect on blastocoelar cell specification. We next examined the effect of blocking Delta-Notch signaling on transfating. Delta MO-injected, PMC(–) embryos were immunostained with MAb 6a9, which recognizes a family of PMC-specific cell surface proteins (MSP130 proteins). We observed large numbers of 6a9-positive cells at the tip of the archenteron at 10 hpd (Fig. 5F), indicating that NSM transfating was not significantly perturbed by the absence of Delta signaling. Also, as in PMC(–) embryos, *alx1* was activated at 2-3 hpd in cells that were located at the tip of the archenteron (Fig. 5E). These findings indicated that in the absence of Delta signaling (and in the absence of pigment cells) transfating was robust and occurred on schedule.

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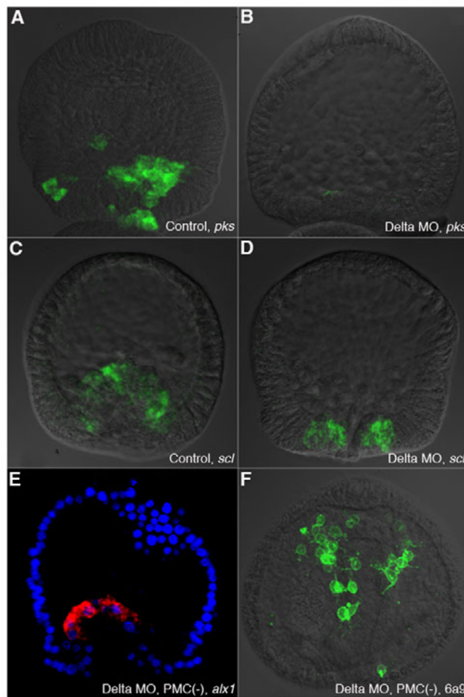

Fig. 5. Disruption of Notch-Delta signaling blocks pigment cell specification but does not affect transfating. (**A-D**) The expression of *pks* and *scl* was examined in sea urchin embryos injected with Delta MO (Delta morphants). *Pks* and *scl* expression in Delta morphants. (A,C) Control embryos analyzed for *pks* (A) and *scl* (C) expression. (B,D) Delta morphants examined for *pks* (B) and *scl* (D) expression. These embryos express *scl* but not *pks*. (**E**,**F**) The activation of the skeletogenic GRN in PMC(–) Delta morphants was assessed by fluorescent whole-mount in situ hybridization (F-WMISH) with *alx1* and immunostaining with MAb 6a9. PMC(–) Delta morphants examined for *alx1* expression at 2 hpd (E; red, *alx1*; blue, Hoechst) and for the presence of 6a9-positive cells (green) at 10 hpd (F). The expression of *alx1* and the 6a9 antigen are unaffected by Delta knockdown. Each image is a *z*-projection of confocal slices and a single differential interference contrast image that was collected at the midpoint of the stack.

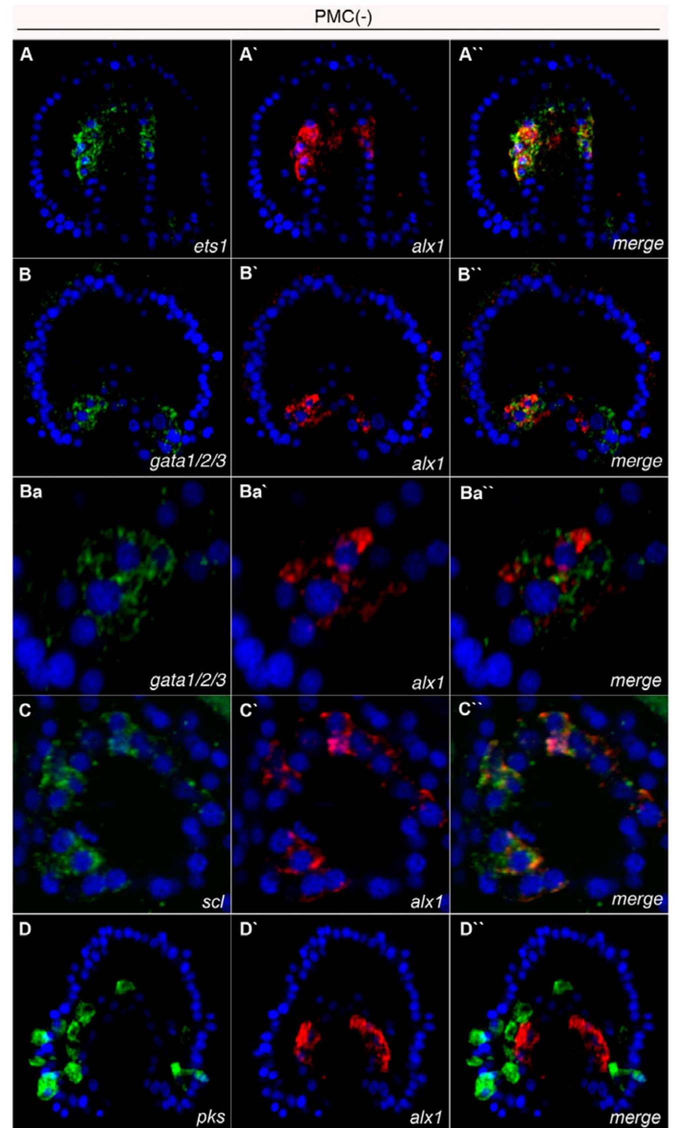

Fig. 6. Presumptive blastocoelar cells transfate following primary mesenchyme cell (PMC) removal. Two-color fluorescent whole-mount in situ hybridization (F-WMISH) was performed on PMC(–) sea urchin embryos using an *alx1* probe (red) and *ets1*, *gata1/2/3*, *scl* or *pks* probes (green). Embryos were counterstained with Hoechst dye (blue). Each panel is a projection of several confocal sections, except images Ba-C″, which are digitally magnified views of a single section. (**A-A″**) Expression of *ets1* (A) and *alx1* (A′) in a PMC(–) embryo at 4 hpd. During transfating, *alx1* is activated in cells that also express *ets1*. (**B-Ba″**) Expression of *gata1/2/3* (B,Ba) and *alx1* (B′,Ba′) in a PMC(–) embryo at 2.5 hpd. *alx1* transcripts are detectable in cells that also express *gata1/2/3*. (**C-C″**) Expression of *scl* (C) and *alx1* (C′) in a PMC(–) embryo at 2.5 hpd. *alx1* transcripts are detectable in cells that also express *scl*. (**D-D″**) *pks* (D) and *alx1* (D′) expression in a PMC(–) embryo at 2.5 hpd. No *alx1* expression is detected in *pks*-positive cells.

Blastocoelar cells leave the tip of the archenteron during gastrulation (Tamboline and Burke, 1992). Two-color F-WMISH analysis shows that *ets1* is ordinarily expressed by blastocoelar cells, but not by pigment cells (T.S. and C.A.E., unpublished observations). In this study, we found that transfating cells co-expressed *alx1* and *ets1*, suggesting that they might be presumptive blastocoelar cells (Fig. 6A-A″). To analyze this further, we cloned the blastocoelar cell markers *gata1/2/3* and *scl* in *L. variegatus*. *gata1/2/3* and *scl* are expressed by presumptive blastocoelar cells at the early mesenchyme blastula stage (Duboc et al., 2010) (data not shown). Because the transfating response begins remarkably quickly (2-3 hpd), we suspected that we might detect the co-expression of *alx1* and *gata1/2/3* (or *scl*) mRNAs in single cells. We performed two-color F-WMISH on PMC(–) embryos at 2.5 hpd using *alx1* and *gata1/2/3* (or *scl*) and, as a control we examined the expression of *alx1* and *pks* in sibling PMC(–) embryos. We found that *alx1* was expressed by cells that also expressed *gata1/2/3* and *scl* (Fig. 6B-C″), but not by *pks*-expressing cells (Fig. 6D-D″). We randomly selected *alx1*-positive cells in these specimens and found that almost all (54/57, 95%) also expressed *scl* or *gata1/2/3*. (Note that *scl* and *gata1/2/3* are co-expressed at this stage; therefore, the expression of either gene implies the expression of both.) These observations indicate that most transfating cells are presumptive blastocoelar cells.

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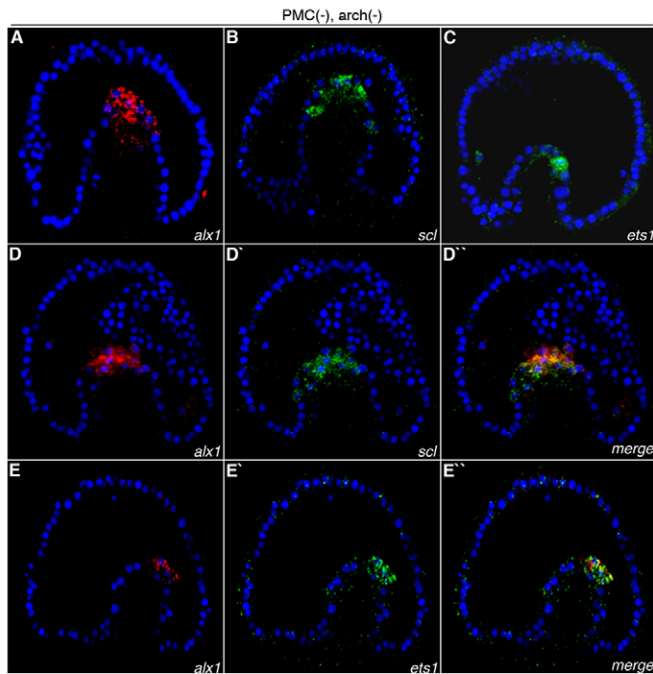

Fig. 7. In PMC(–), arch(–) embryos the skeletogenic gene regulatory network (GRN) is activated in cells that re-establish a blastocoelar cell-like fate. (**A**) *alx1* expression (red) in a PMC(–), arch(–) sea urchin embryo. *alx1* is activated 7-8 hours after archenteron removal, in cells at the tip of the regenerating archenteron. (**B**,**C**) *Scl* and *ets1* (green) expression in a PMC(–), arch(–) embryo. These blastocoelar markers are also expressed in cells at the tip of the archenteron. (**D-E″**) Two-color fluorescent whole-mount in situ hybridization (F-WMISH) for *scl* and *ets1* (green) in combination with *alx1* (red) shows that *alx1* is expressed in cells that also express *scl* (D-D″) and *ets1* (E-E″). Embryos were counterstained with Hoechst dye (blue).

Endoderm transfating involves the re-establishment of a blastocoelar cell-like state and delayed activation of the PMC GRN

Endoderm cells are conditionally specified and have the capacity to activate the skeletogenic program following the microsurgical removal of the PMCs and the archenteron, which includes the NSM territory (McClay and Logan, 1996). We refer to such embryos as PMC(–), arch(–) embryos. At present, nothing is known concerning the deployment of the PMC GRN in such embryos. To determine the timing of activation of the PMC GRN, we first examined the expression of *alx1* in PMC(–), arch(–) embryos at various times after archenteron removal. *alx1* was first expressed 7-8 hours after surgery in cells that were located at the tip of the archenteron (Fig. 7A). Thus, there is significant delay (~5 hours) in the deployment of the PMC GRN during endoderm transfating compared with the activation of the network during NSM transfating.

We also examined the expression of *scl* and *ets1* in PMC(–), arch(–) embryos and found that the expression of these blastocoelar cell markers was also re-established at the tip of the archenteron (Fig. 7B,C). To test directly whether *scl*- and *ets1*-expressing cells were the cells that activated *alx1* during endodermal transfating, we performed two-color F-WMISH, and found that the *scl*- and *ets1*-positive cells also expressed *alx1* (Fig. 7D″,E″). We conclude that during endoderm transfating cells at the tip of the archenteron re-establish a blastocoelar cell-like regulatory state and that these same cells activate the PMC GRN.

The role of MAPK signaling differs in regulative and normal development

During normal development, the MAPK signaling pathway is required for PMC ingression and for maintaining the expression of *alx1* in the large micromere-PMC lineage (Röttinger et al., 2004; Sharma and Ettensohn, 2010). We tested the role of MAPK signaling in controlling the expression of another early regulatory gene in the PMC GRN, *tbr*. We found that, as in the case of *alx1*, there was robust activation of *tbr* in embryos that were treated continuously from the two-cell stage with the MEK-inhibitor U0126, but by the pre-ingression blastula stage, *tbr* transcripts were no longer detectable by F-WMISH (see Fig. S2 in the supplementary material). We also observed that in the presence of U0126, downregulation of *alx1* transcripts occurred earlier than downregulation of *tbr* transcripts; this difference might reflect a higher abundance or a greater stability of *tbr* transcripts.

Previous studies have shown that MAPK signaling is required for the process of transfating (Ettensohn et al., 2007). We looked more closely at the role of the MAPK pathway during transfating, focusing on the initial phase of activation of *alx1* and *tbr*. In control PMC(–) embryos, *alx1* and *tbr* expression was detected by 2 hpd (6/6 embryos; Fig. 8A,A′). In PMC(–), U0126-treated embryos, however, the activation of *alx1* and *tbr* was suppressed (4/5 embryos showed no detectable expression in any cell, 1/5 showed a greatly reduced number of positive cells; Fig. 8B,B′). We also confirmed that *alx1* and *tbr* were not expressed at 4 hpd (10/10 embryos lacked expression in any cell; Fig. 8D,D′), indicating that the inhibitor did not simply delay the activation of these genes. These findings point to a significant difference in the role of MAPK signaling in the skeletogenic GRN as it is deployed in the large micromere-PMC lineage and in transfating NSM cells. During normal development, MAPK signaling is required for the maintenance, but not for the activation, of the GRN. By contrast, our inhibitor studies revealed no MAPK/*ets*-independent mechanisms of GRN activation in NSM cells; instead, MAPK signaling is required for the initial deployment of the network. We found that the expression of *ets1* itself was not affected in PMC(–), U0126-treated embryos (Fig. 8E,F).

To test whether the MAPK signaling pathway is also essential for activating the PMC GRN during endoderm cell transfating, PMC(–), arch(–) embryos were treated with U0126 and the expression of *alx1* was analyzed. We found that *alx1* was not expressed in these embryos (4/5 embryos showed no expression, 1 embryo had a single labeled cell; Fig. 9D), whereas all control (sibling, DMSO-treated) PMC(–), arch(–) embryos showed robust expression (Fig. 9C). U0126-treated PMC(–), arch(–) embryos also did not secrete a larval skeleton (Fig. 9B). These results indicate that the MAPK pathway is also required for the activation of the PMC GRN during endoderm transfating, in contrast to its role during normal development.

Cell division is not required for transfating by NSM or endoderm cells

We incubated embryos with EdU, a thymidine analog, to determine whether NSM cells divide during transfating. Transfated NSM cells were identified 7-11 hours after PMC removal by immunostaining with MAb 6a9. Under these conditions, most 6a9-positive cells (150/229, 66%) were not labeled with EdU, indicating that they had fully deployed the skeletogenic network in the absence of DNA synthesis and cell division (Fig. 10A,A′). Some 6a9-positive, EdU-positive cells probably underwent mitosis during the course of the experiment;

DEVELOPMENT

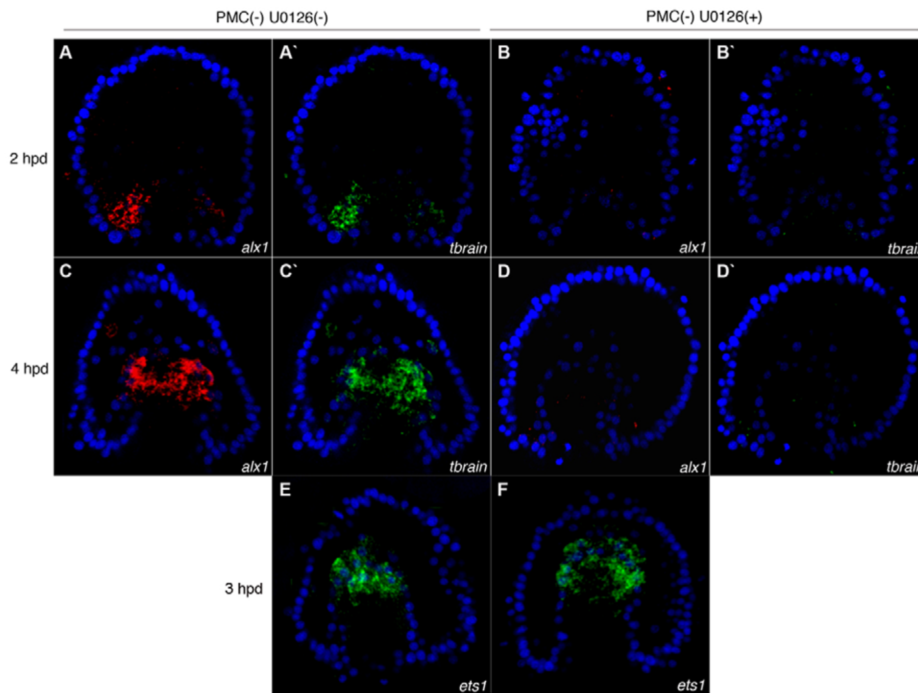

Fig. 8. The role of MAPK signaling differs in regulative and normal development. The expression of *alx1* (red) and *tbr* (green) was examined in PMC(–) sea urchin embryos that were treated with U0126 and in DMSO-treated sibling PMC(–) embryos at 2 and 3 hpd. Each image is a projection of several confocal slices. (**A**,**A′**,**C**,**C′**) Control PMC(–) embryos at 2 hpd (A,A′) and 3 hpd (C,C′) showing the activation of *alx1* and *tbr* during transfating. (**B**,**B′**,**D**,**D′**) PMC(–) embryos at 2 hpd (B,B′) and 3 hpd (D,D′) showing the absence of *alx1* and *tbr* activation in presence of U0126. (**E**,**F**) The expression of *ets1* in PMC(–), U0126-treated and DMSO-treated, sibling PMC(–) embryos at 3 hpd. In PMC(–) control (E) and PMC(–), U0126-treated embryos, *ets1* is expressed by the transfating cells. Embryos were counterstained with Hoechst dye (blue).

therefore, the actual fraction of NSM cells that were present at the time of PMC removal and which activated the skeletogenic GRN without dividing was presumably greater than 66%. Because NSM cells and PMCs are both derived from mesoderm, we also asked whether cell division might be required for more extensive GRN reprogramming; i.e. across a germ layer boundary. EdU labeling of PMC(–), arch(–) embryos showed that the majority of endoderm cells that deployed the GRN under these conditions did not undergo DNA synthesis (Fig. 10B,B′). 12 hours after NSM removal, 65/83 (78%) of 6a9-positive cells were not labeled with EdU. To confirm that cell division was not required for transfating, we treated embryos with aphidicolin, an inhibitor of DNA polymerase I that blocks DNA synthesis and cell division in sea urchin embryos (Stephens et al., 1986). We observed a robust transfating response in PMC(–) embryos (Fig. 10C) and PMC(–), arch(–) embryos (data not shown), despite the inhibition of DNA synthesis, which was indicated by a lack of EdU labeling throughout the embryo.

DISCUSSION

In the sea urchin embryo, maternal factors and differential zygotic gene expression partition the embryo into distinct transcriptional domains very early in development. The transcriptional networks that are deployed during early development are relatively shallow and lead rapidly to the regional expression of terminal differentiation genes in various embryonic territories. Despite these early patterning processes, genomic regulatory programs are not fixed and many cells remain developmentally labile, even quite late in development. In this study, we have taken advantage of the recent elucidation of GRNs in the sea urchin embryo (in particular, the well-defined micromere-PMC GRN) to address questions related to developmental plasticity and genomic reprogramming.

During normal development, the skeletogenic GRN is activated by maternally entrained mechanisms that operate autonomously within the large micromere-PMC lineage (Fig. 11). The local stabilization of β-catenin in the vegetal region of the embryo directly activates *pmar1* and, because Pmar1 is a repressor, it presumably activates the GRN by blocking the expression of a second repressor (Oliveri et al., 2002). One target of Pmar1 is the repressor *hesC* (Revilla-i-Domingo et al., 2007). The repression of *hesC* does not account for the initial activation of the PMC GRN, however, as the level of *hesC* mRNA does not decline in the large micromere territory until after the network has been activated there (Sharma and Ettensohn, 2010). It is likely, therefore, that additional local activators and/or repressors are involved. We have also shown that the expression of two early genes, *alx1* and *delta*, but not that of *pmar1*, is dependent on unequal cell division (Sharma and Ettensohn, 2010).

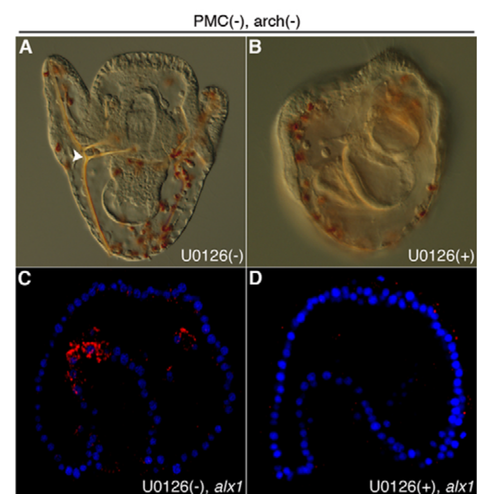

Fig. 9. Endodermal cell transfating requires MAPK signaling. (**A**) Control PMC(–), arch(–) embryo at 48 hpf. The arrowhead points to the skeleton. (**B**) Sibling U0126-treated PMC(–), arch(–) embryo. U0126-treated embryos fail to form skeletal rods. (**C**,**D**) Alx1 expression (red) in PMC(–), arch(–) embryos, 8 hours after archenteron removal. Control (DMSO-treated) embryos show normal expression of *alx1* (C) but sibling, U0126-treated embryos fail to activate *alx1* (D). Embryos were counterstained with Hoechst dye (blue) in C,D.

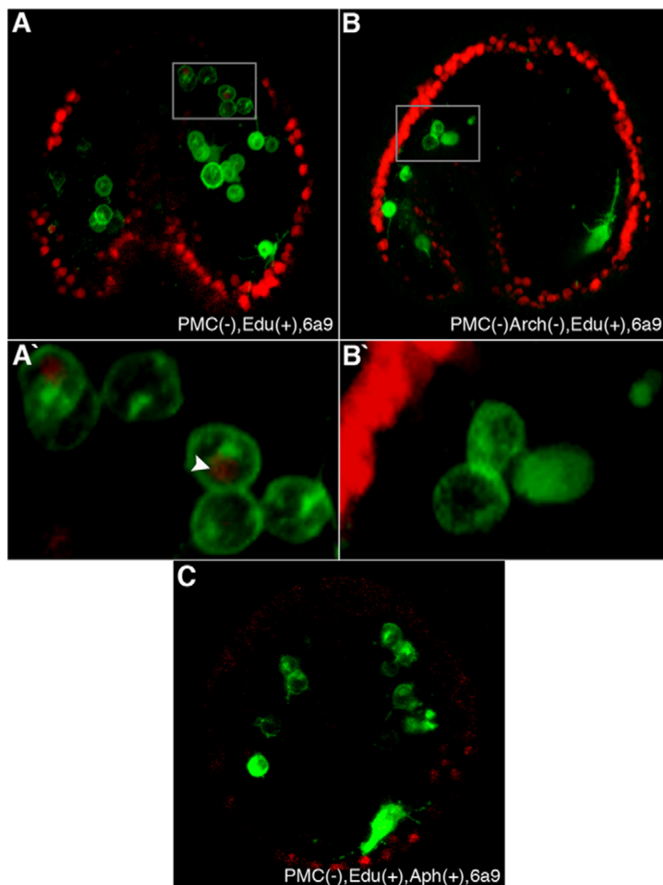

Fig. 10. Cell division is not required for non-skeletogenic mesoderm (NSM) or endoderm transfating in sea urchin. MAb 6a9 immunostaining is shown in green. (**A**) An EdU treated, PMC(–) embryo at 9 hpd. Most transfated cells lack nuclear EdU label (red). (**A′**) Magnified view of the boxed area in panel A. The arrowhead points to a transfated cell that has incorporated EdU label (red). (**C**) An aphidicolin + EdU treated, PMC(–) embryo at 9 hpd. NSM cells transfate to a skeletogenic fate in the absence of DNA synthesis and cell division. (**B**) An EdU-treated PMC(–), arch(–) embryo at 12 hpd. Most transfated cells lack nuclear EdU label. (**B′**) Magnified view of the boxed area in panel B.

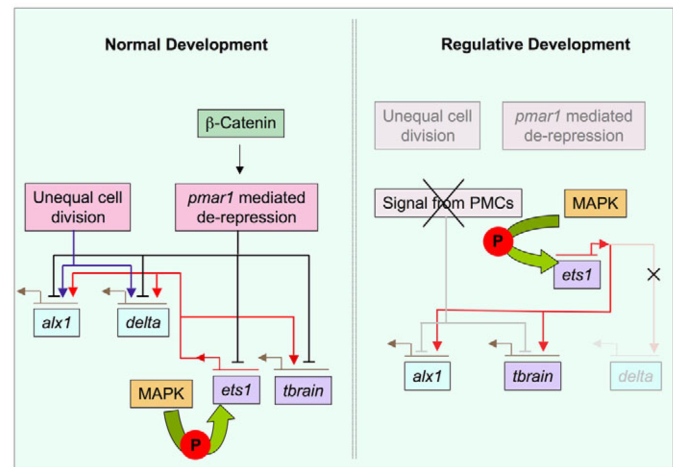

Fig. 11. Summary of differences in the skeletogenic gene regulatory network (GRN) during normal and regulative development. The skeletogenic GRN during transfating is activated by mechanisms that are independent of *pmar1* and unequal cell division. The regulative activation of the network is dependent on MAPK signaling, which probably mediates the phosphorylation of Ets1, but there is no evidence of MAPK-independent activation mechanisms. In contrast to normal development, *alx1* and *tbr* are activated simultaneously and *delta* is not activated. Straight arrows and T-shaped bars represent activation and inhibition, respectively. Curved arrows represent phosphorylation. PMC, primary mesenchyme cell.

These various inputs lead to the activation of a core set of early genes, which includes *alx1*, *tbr*, *ets1* and *delta*. Although these genes are usually considered to have a common mechanism of activation, it appears that they are not expressed synchronously in the large micromere territory; a variety of evidence indicates that the zygotic activation of *tbr* follows that of *alx1* and *delta* (Croce et al., 2001; Croce and McClay, 2010; Ettensohn et al., 2003; Fuchikama et al., 2002; Ochiai et al., 2008). Our multiplex F-WMISH analysis confirmed that, in *L. variegatus*, the accumulation of *alx1* mRNA in the large micromere territory precedes that of *tbr* mRNA.

In striking contrast to the deployment of the network during normal development, the activation of the GRN in NSM cells (and endoderm cells) is tightly regulated by extrinsic signals and is independent of *pmar1* (Ettensohn et al., 2007). The present study has revealed several additional differences in the upstream regulation of the network in transfating NSM cells (Fig. 11). There is a shift in the relative timing of expression of *alx1* and *tbr*, a finding that points to possible changes in the upstream regulation of the network during transfating. Moreover, we find that *delta* is not activated by transfating NSM cells. The role of micromere-derived Delta is to specify the overlying NSM, a function that is likely to be irrelevant at the stage when the process of NSM transfating is initiated. One hypothesis is that the loss of *delta* expression in the NSM, which normally occurs by the early gastrula stage (Sweet et al., 2002), occurs by mechanisms that are irreversible; therefore, *delta* might no longer respond to the same inputs that ordinarily activate this gene in the micromere-PMC lineage. An alternative hypothesis, however, is that some or all of the inputs that ordinarily coordinate the activation of *alx1*, *tbr*, *ets1* and *delta* in the micromere territory are not employed during transfating, and therefore these genes are no longer subject to parallel regulation.

The MAPK signaling pathway provides essential inputs into the micromere-PMC GRN. This pathway is believed to result in the phosphorylation of Ets1, which is required for the later expression (but not for the initial activation) of *alx1* and *tbr* in the micromere territory during normal development (Röttinger et al., 2004; Sharma and Ettensohn, 2010) (this study). By contrast, we have found that MAPK signaling is required for the activation of both *alx1* and *tbr* in transfating NSM and endoderm cells. Inhibition of MAPK signaling does not affect the expression of *ets1*, either in the large micromere-PMC lineage (Röttinger et al., 2004) or in transfating NSM cells (this study), a finding which supports the view that MAPK signaling acts downstream of *ets1* transcription. Overall, our results suggest that phosphorylated Ets1 provides an essential, early input into *alx1* and *tbr* in transfating NSM cells, whereas its role during normal development is to provide a late input that positively regulates the expression of these genes. Although the relative contribution of the Ets1 input to the deployment of the network differs in the two scenarios, the molecular nature of the input might, in fact, be the same (e.g. in both scenarios, phosphorylated Ets1 might positively regulate the network by binding to the same cis-regulatory modules of *alx1* and *tbr*).

Gene epistasis studies and/or cis-regulatory analyses of *alx1*, *tbr* and *delta* have identified positive inputs from Ets1 and negative inputs from HesC (Ochiai et al., 2008; Revilla-i-Domingo et al., 2007; Smith and Davidson, 2008; Wahl et al., 2009). During gastrulation, *ets1* is expressed in the NSM territory and *hesC* is silent, yet *tbr* and *alx1* are not ordinarily expressed by NSM cells. Moreover, the Ets1 protein that is produced is concentrated in the nuclei of NSM cells (C.A.E., unpublished observations) and is probably phosphorylated, as ERK is active in the NSM territory (Röttinger et al., 2004; Rizzo et al., 2006). Wahl and co-workers have suggested that, in NSM cells, Erg competes with Ets1 for binding to the same DNA target sites but lacks an activation function; this might not occur in PMCs if levels of Erg are too low (Wahl et al., 2009). Thus, the network might be activated in NSM cells via a double-repression mechanism, whereby Erg (or a different repressor) is inactivated following the loss of the PMC-derived signal. Many other models may be envisaged, however.

Whatever regulatory mechanisms are responsible for the activation of *alx1* and *tbr* in the NSM territory during transfating, it is evident that they are deployed quite rapidly. It was previously reported that *alx1* expression is detectable in NSM cells 3-4 hpd (Ettensohn et al., 2007). In this study, using a more sensitive method, we have documented the accumulation of *alx1* transcripts in NSM cells 2-3 hpd. *alx1* is a relatively large gene (~37 kb) and, following the activation of *alx1* transcription, ~40 minutes would be required for the appearance of the first complete transcript, assuming a transcription rate of 900 nt/minute at 24°C (Ben-Tabou de-Leon and Davidson, 2009). Thus, *alx1* transcription is probably initiated less than 2 hours after PMC removal.

Analysis of a set of downstream genes in the PMC GRN, which includes the late regulatory genes *dri*, *jun* and *foxB*, and the tyrosine kinase receptors *vegfr-Ig-10* and *fgfr-2*, reveals that these genes are activated in transfating NSM cells in a temporal sequence that resembles their order of activation during normal development. These findings support the view that, despite differences in the upstream inputs into the network, and differences in the regulatory states of PMCs and NSM cells at the time that the network is activated, the later regulatory layers of the skeletogenic GRN are fully recapitulated during NSM transfating. The faithful deployment of the downstream layers of the network explains the remarkable extent to which the morphogenetic behaviors of transfated NSM cells mimic those of PMCs. For example, during transfating, NSM cells become competent to respond to PMC-specific migratory guidance cues. Our finding that transfating cells activate the expression of *vegfr-Ig-10* and *fgfr-2*, two receptors that have recently been implicated in PMC migration and guidance (Duloquin et al., 2007; Röttinger et al., 2008), partly explain these dramatic changes in cell behavior.

Pigment and blastocoelar cells are the two principal populations of migratory NSM cells. A previous study suggested that the subpopulation of NSM cells that transfates might be presumptive pigment cells (Ettensohn and Ruffins, 1993). Owing to the lack of molecular markers at that time, this finding was based solely on a ~50% reduction in the numbers of pigment cells in PMC(–) embryos at the pluteus larva stage. In this study, using molecular markers for pigment and blastocoelar cells, and focusing specifically on the initial stages of transfating, we show that the great majority of cells that transfate following PMC removal are *scl*(+), *gata1/2/3*(+) cells that lie on the oral (ventral) side of the archenteron; i.e. cells that would otherwise give rise predominantly to blastocoelar cells. One possible explanation for the reduced numbers of pigment cells in PMC(–) larvae is that mitotic divisions of pigment cells that occur after ingression are perturbed in some way by PMC removal. Another possibility is that some of the cells in PMC(–) embryos that express *pks* at the gastrula stage might fail to develop pigment at later stages, when Ettensohn and Ruffins (Ettensohn and Ruffins, 1993) counted pigment cells.

Blastocoelar cells and PMCs exhibit similar morphogenetic behaviors, including EMT, filopodia-based motility, and cell-cell fusion. Several regulatory genes of the PMC GRN are also ordinarily expressed by blastocoelar cells, including members of the *ets* family (*ets1*, *erg* and *ese*) (Rizzo et al., 2006; Röttinger et al., 2004), the forkhead family (*foxN2/3* and *foxO*) (Tu et al., 2006) and *snail* (Wu and McClay, 2007). We have recently identified many extracellular matrix proteins and cytoskeletal proteins that are selectively co-expressed by these two cell types (C.A.E., unpublished observations). These observations point to striking similarities in the molecular programs of PMCs and blastocoelar cells and suggest that they share elements of a common mesenchymal regulatory state. The regulatory states of the two cell types are distinct in other respects, however. For example, *foxa*, *gcm*, *scl* and *gata1/2/3* are all expressed in presumptive blastocoelar cells prior to gastrulation, but these genes are never expressed in the large micromere territory.

McClay and Logan (McClay and Logan, 1996) showed that presumptive endoderm cells have the capacity to activate the PMC GRN. We have confirmed that PMC removal, followed by removal of the NSM territory, induces the ectopic activation of the skeletogenic GRN in a subset of presumptive endoderm cells. *alx1*, an early marker, accumulates in cells near the tip of the regenerated archenteron, but in a delayed fashion compared with PMC(–) embryos, a delay that might reflect a more extensive genomic reprogramming. Our findings show that activation of the GRN by endoderm cells occurs via the regeneration of an NSM territory, by mechanisms that are unknown. During the regeneration process, endoderm cells re-establish at least some elements of a blastocoelar cell regulatory state, as shown by the de novo activation of *scl* and *ets1*. The activation of *alx1* in transfating endoderm cells, as in transfating NSM, is dependent on MAPK signaling and probably acts via Ets1 phosphorylation. These findings highlight the fact that the same GRN circuitry can be fully deployed within the context of multiple, pre-existing cell regulatory states.

Cell division has been proposed to play an important role in facilitating genomic reprogramming. Many transcription factors, including RNA polymerase II, are released from chromatin during mitosis, which might promote reprogramming (Egli et al., 2008). Nuclear envelope disassembly/reassembly might allow global changes in nuclear architecture that alter patterns of gene expression (Reddy et al., 2008). An early step in the conversion of somatic cells into induced pluripotent stem (iPS) cells is the acquisition of a program of rapid division (Smith et al., 2010), and experimental manipulation of cell cycle regulators indicates that proliferation is required for iPS cell formation (Ruiz et al., 2011). On the other hand, substantial reprogramming of somatic cell nuclei occurs in heterokaryons in the absence of DNA synthesis and cell division (Bhutani et al., 2010).

Our findings show that most transfating cells do not undergo mitosis during their reprogramming to a PMC-like state. In the case of NSM cells, this finding is consistent with the rapid deployment of the GRN (this study), and the relatively long average cell cycle time at the gastrula stage (>6 hours in *L. variegatus*) (Nislow and Morrill, 1988). Surprisingly, even in the case of the slower (and presumably more extensive) reprogramming of endoderm, a large majority of the cells do not undergo mitosis during the transfating process. These findings also show that unequal cell division, which plays a pivotal role in activating the skeletogenic GRN in the

micromere-PMC lineage during normal development (Sharma and Ettensohn, 2010), is not required for the regulative activation of the GRN. This is consistent with the view that the unequal division of vegetal blastomeres, and the linkages between this pattern of division and GRN activation, are recent evolutionary inventions (Ettensohn, 2009). More generally, our findings show that, at least in the context of the reprogramming of developmental GRNs, the dissociation of transcription factors from DNA, or other changes in nuclear organization during mitosis, do not play a crucial role in the reprogramming process.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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