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The Snail repressor is required for PMC ingression in the sea urchin embryo

Shu-Yu Wu and David R. McClay*

In metazoans, the epithelial-mesenchymal transition (EMT) is a crucial process for placing the mesoderm beneath the ectoderm. Primary mesenchyme cells (PMCs) at the vegetal pole of the sea urchin embryo ingress into the floor of the blastocoele from the blastula epithelium and later become the skeletogenic mesenchyme. This ingression movement is a classic EMT during which the PMCs penetrate the basal lamina, lose adherens junctions and migrate into the blastocoele. Later, secondary mesenchyme cells (SMCs) also enter the blastocoele via an EMT, but they accompany the invagination of the archenteron initially, in much the same way vertebrate mesenchyme enters the embryo along with endoderm. Here we identify a sea urchin ortholog of the Snail transcription factor, and focus on its roles regulating EMT during PMC ingression. Functional knockdown analyses of Snail in whole embryos and chimeras demonstrate that Snail is required in micromeres for PMC ingression. Snail represses the transcription of cadherin, a repression that appears evolutionarily conserved throughout the animal kingdom. Furthermore, Snail expression is required for endocytosis of cadherin, a cellular activity that accompanies PMC ingression. Perturbation studies position Snail in the sea urchin micromere-PMC gene regulatory network (GRN), downstream of Pmar1 and Alx1, and upstream of several PMC-expressed proteins. Taken together, our findings indicate that Snail plays an essential role in PMCs to control the EMT process, in part through its repression of cadherin expression during PMC ingression, and in part through its role in the endocytosis that helps convert an epithelial cell to a mesenchyme cell.

KEY WORDS: Snail, Primary mesenchyme cell, Ingression, Epithelial-mesenchymal transition, Gene regulatory network

INTRODUCTION

At the onset of gastrulation, micromere progeny of the sea urchin embryo undergo a phenotypic shift from epithelial cells to migratory primary mesenchyme cells (PMCs) via ingression, an example of an epithelial-mesenchymal transition (EMT). These nascent PMCs ingress through the basal lamina and migrate into the blastocoele (for details, see Katow and Solursh, 1980) (reviewed by Solursh, 1986). During EMT, the PMCs downregulate cell-cell adhesion (Fink and McClay, 1985), increase motility, change cell shape, and finally become mesenchymal cells with a migratory behavior. These PMCs exclusively form the larval skeleton later in development.

Micromeres, the PMC predecessors, appear at fourth cleavage as a result of an unequal cleavage in the vegetal hemisphere. During early cleavage, they become autonomously specified (Horstadius, 1973; Okazaki, 1975; Davidson et al., 1998; Ransick and Davidson, 1993), and this specification system is coupled to the early establishment of the animal-vegetal axis in the unfertilized egg (Angerer and Angerer, 2003; Brandhorst and Klein, 2002; Ettensohn and Sweet, 2000). Although the responsible maternal determinants initiating this event are incompletely understood, one of the earliest known components is β-catenin-induced transcriptional activation at fourth cleavage (Logan et al., 1999). The nuclear localization of β-catenin is required for micromere specification and later for all endomesoderm formation, including archenteron and secondary mesenchyme cells (SMCs) (Emily-Fenouil et al., 1998; Logan et al., 1999; Wikramanayake et al., 1998). β-catenin provides an early input into the PMC gene regulatory network (GRN), where it activates a transcriptional repressor *pmar1*, the earliest known zygotic gene expressed exclusively in the micromere lineage (Oliveri et al., 2002). Pmar1 protein activates the micromere specification program by repressing an unidentified, ubiquitous repressor. As a consequence of this derepression event, several downstream transcriptional regulators are activated, among which are the zygotic targets, *alx1* (Ettensohn et al., 2003) and *ets1* (Kurokawa et al., 1999). When the function of either Alx1 or Ets1 is blocked, PMC specification is disrupted. Despite the increasing knowledge of the mechanisms of PMC specification, much less is known about what triggers the EMT process in these cells and what molecular changes are required for the morphogenesis of PMCs [as suggested also in Shook and Keller (Shook and Keller, 2003)].

The Snail gene family of transcription factors has been shown to play crucial roles in mesoderm development, cell movement and especially induction of EMT in other systems (Barrallo-Gimeno and Nieto, 2005; Hemavathy et al., 2000; Nieto, 2002). Although originally identified as a mesoderm determinant in *Drosophila* (Alberga et al., 1991), the first indication that the Snail family is involved in EMT came from *Snail2* (*Slug*) loss-of-function studies in chick embryos. Incubation of early chick embryos with antisense oligonucleotides to inhibit *Snail2* function led to the failure of early mesoderm migration from the primitive streak (Nieto et al., 1994). Subsequent studies in cell lines and in other vertebrates later confirmed this involvement. For example, mouse *Snail* (*Snai1* – Mouse Genome Informatics) is able to induce EMT when expressed in mammalian epithelial cells (Batlle et al., 2000; Cano et al., 2000), and *Snail*-knockout mice die during gastrulation, due at least in part, to the failure of the mesodermal cells to undergo an EMT (Carver et al., 2001). Furthermore, different signaling molecules, such as TGFβ, FGFs, EGFs, WNTs, BMPs and Notch, have been shown to

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trigger EMT processes by inducing Snail gene expression in different cellular contexts (reviewed by De Craene et al., 2005). The PMC ingression of the sea urchin embryo is an excellent model for studying EMT in vivo, and with the recently published sea urchin endomesoderm GRN (Davidson et al., 2002a; Davidson et al., 2002b), we asked whether Snail has the same role in the sea urchin as in other systems exhibiting EMTs. If so, how did it fit into the micromere GRN?

In this study, we report the identification, characterization, and functional analysis of *Lvsnail*, a member of Snail family transcription factors in *Lytechinus variegatus*. *Lvsnail* mRNA is expressed dynamically in different mesodermal cell populations throughout the development of the sea urchin embryo. We show that LvSnail is required for the micromeres to undergo EMT, and ingress into the blastocoele. Moreover, LvSnail mediates the downregulation of cadherin expression and function. We further position *Lvsnail* in the current version of the micromere GRN, and examine its regulative relationships with several PMC genes.

MATERIALS AND METHODS

Animals

L. variegatus adults were obtained from Florida (Sea Life, Tavernier, FL), or from the Duke University Marine Laboratory at Beaufort, NC. Gametes were harvested, cultured and injected by standard methods.

Cloning of *Lvsnail* and *Lvpmar1*

The coding sequence of *Lvsnail* was obtained by RT-PCR from a *Lytechinus variegatus* gastrula cDNA library (GenBank Accession Number DQ665364). The sequence information of *Lvpmar1* (Lv_170H13; Accession Number DQ667003) was obtained from Caltech *Lytechinus variegatus* sperm genomic BAC library A, as described by Davidson et al. (Davidson et al., 2002b), and the full open reading frame was subsequently amplified by PCR from Lv cDNA libraries. All PCR products were cloned into a PCS2 vector for mRNA synthesis.

In situ hybridization

In situ hybridization was performed using standard methods (Bradham and McClay, 2006) with DIG-labeled RNA probes and BM purple substrate (Roche) for detection. Hybridizations and washes were carried out at 65°C. The *Lvsnail* probe corresponds to the full-length open reading frame. Other probes were synthesized from *Lvets1* (this study) and *Lvalx1* (Ettensohn et al., 2003) clones.

Morpholino antisense oligonucleotides (MASO), mRNA injections, and U0126

Two *Lvsnail*-specific MASOs were obtained from Gene Tools, although Oligo2 had a higher efficiency at 1 mM, (Oligo1: 5′-AAAGACCCTC-GGCATCTTCTTGATAA-3′; Oligo2: 5′-TTTTGACGAGAAAAGACC-TCGGCAT-3′). Alx1MASO was injected at 2 mM (see Ettensohn et al., 2003). Each injected mRNA was transcribed in vitro using the mMessage mMachine Kit (Ambion), and diluted in ddH2O. A final concentration of 25-30 ng/μL was used for *pmar1* mRNA, and 400-500 ng/μL for *snail* mRNA. Double injections were performed by simultaneous injections of *pmar1* mRNA plus SnaMASO or Alx1MASO plus *snail* mRNA at the concentrations indicated above. CadTM-GFP was constructed by linking the transmembrane domain of LvG-Cadherin in between two full-length GFPs. U0126 (Promega) was added to cultures during early cleavage stages, unless otherwise noted. Treatment with the DMSO vehicle had no effect (not shown). Doses for these reagents were determined by dose-response experiments. U0126 was used at 10-15 μM in most experiments.

Transplantation experiments

Animal-vegetal half transplantations were performed at the 16- or 32-cell stage, and micromere transplantations were performed at 16-cell stage, with *L. variegatus* embryos. Detailed procedures were followed as previously described (Logan et al., 1999).

Immunostaining

Embryos were methanol-fixed, stained with 1d5 mAb (1:200) in 4% normal goat serum in PBS, and incubated overnight at 4°C. After washing three times in PBS, samples were incubated with Cy3-conjugated secondary antibodies (Jackson Immunoresearch Laboratories) for 1-2 hours at room temperature, and then imaged as previously described (Gross et al., 2003).

QPCR analysis

Total RNA was prepared from 10-20 embryos using Trizol (Invitrogen) with a glycogen carrier (Ambion). The sample was used for reverse transcription (RT) with Taqman RT-PCR kits (Applied Biosystems) after pretreatment with DNase I (DNA-free, Ambion). QPCRs were performed using Roche LightCycler and a Fast Start SYBR Green PCR Kit (Roche). Results were calculated by subtracting the sample CT (crossing point threshold) from the control CT to determine ΔCT, and then normalized to Ubiquitin.

RESULTS

Cloning and sequence analysis of *Lvsnail*

Snail belongs to the zinc-finger transcription factor family, which is highly conserved in the zinc-finger domain. Using RT-PCR, the open reading frame of *Lvsnail* was amplified and cloned from a late-gastula stage cDNA pool (Accession Number DQ665364). *Lvsnail* encodes a 337 amino acid protein based on the primary sequence data. ClustalW alignment between LvSnail and SpSnail proteins (*Strongylocentrotus purpuratus*, Accession Number AY372519) shows the two proteins share an overall amino acid identity of 92%, despite separation of about 30-40 million years.

Proteins in the *snail* gene family possess 4-6 C_2H_2 zinc-fingers (Nieto, 2002) as the DNA binding domain at the C-terminus. LvSnail has five zinc-fingers, and the protein alignment from ZFII to ZFV (the first zinc-finger is missing in human, mouse and zebrafish) shows that this region is highly conserved relative to *snail* family members from other species (Fig. 1A) [e.g. Slug (Snail2) from vertebrates (88-89% amino acid identity) and Snail from amphioxus (87%)]. In addition, Snail family proteins are characterized by the presence of an N-terminal SNAG domain (nine amino acids). Again, the SNAG domain of LvSnail is almost identical to all Snail family members from other species except *Drosophila*, *Caenorhabditis elegans* and *Ciona* (Fig. 1B), which may be due to independent losses in these lineages.

Phylogenetic analysis based on the four zinc-finger regions, using the neighbor-joining method, groups LvSnail and SpSnail together into a large clade containing *snail* and *slug* genes from vertebrates, amphioxus *snail* (Bf *snail*) and limpet *snail* (Pv *snail2*) (Fig. 1C). The topology of this phylogenetic tree, as well as the distinct protein motifs, clearly support the conclusion that *Lvsnail* is an ortholog of *snail* genes.

Lvsnail mRNA is expressed dynamically in mesoderm during gastrulation

A temporal expression profile of *Lvsnail* mRNA was obtained using quantitative PCR (data not shown). Although the expression level of *Lvsnail* remained relatively low throughout all stages, there was no maternal message, and zygotic *Lvsnail* mRNA transcripts began to accumulate around the hatched blastula stage, and then increased until prism stage.

Whole-mount in situ hybridization (WMISH) showed that *Lvsnail* mRNA is expressed dynamically in different territories of the embryos (Fig. 2). No staining appears in early cleavage stages (Fig. 2A), and expression is first detectable around the late-hatched blastula (HB) in the thickened vegetal plate region (Fig. 2B). At early mesenchyme blastula (MB) stage, *Lvsnail* mRNA is expressed in ingressing PMCs (Fig. 2C). The PMC expression of *Lvsnail* is reduced following ingression, and staining then appears within the SMC territory during

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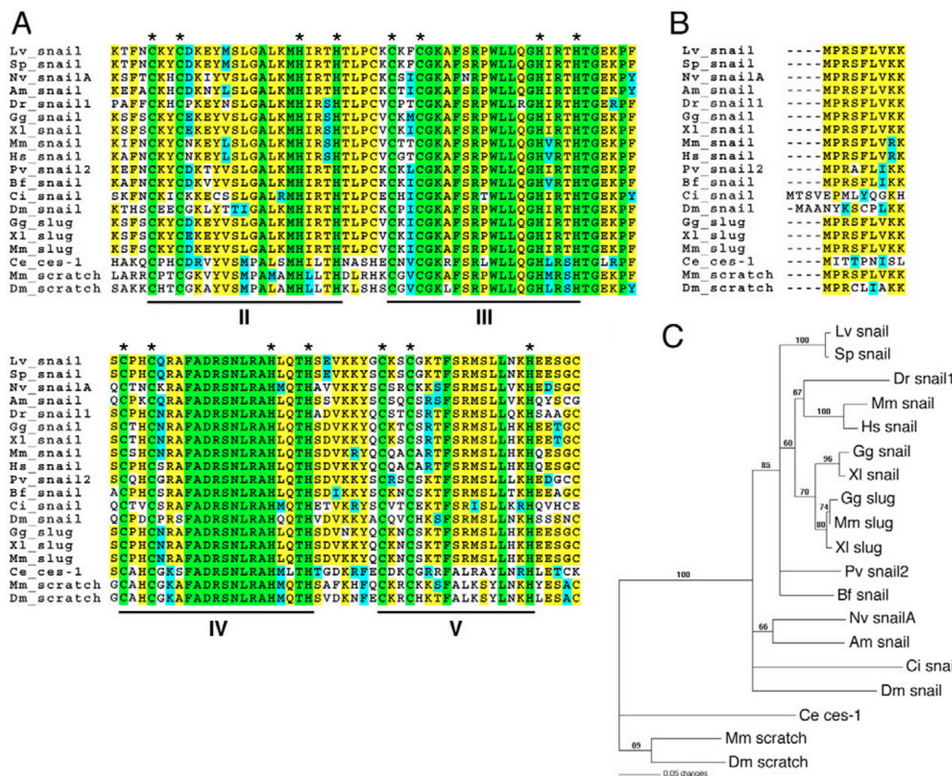

Fig. 1. Sequence comparisons of *Lytechinus* Snail and related Snail family proteins. (**A**) The zinc finger region of *Lytechinus* Snail compared with related proteins in other organisms. Positions of zinc-finger II-V are shown and the conserved cysteines and histidines are indicated with asterisks. (**B**) The SNAG domain of *Lytechinus* Snail fits the consensus and is identical to those of several other *snail* family members, including *Acropora* Snail and mouse Slug and Scratch. (**C**) Rooted neighbor-joining tree showing the relationship of *Lytechinus* Snail with other *snail* family proteins (1000 bootstraps, values indicated on nodes). Mouse and *Drosophila* Scratch served as outgroups.

archenteron invagination, and prior to ingression of SMCs (Fig. 2D), then disappears from the ingressed SMCs. Throughout gastrulation, the SMC expression of *Lvsnail* persists at the tip of archenteron (Fig. 2E), and exhibits both oral-aboral and left-right asymmetry at the mid-late gastrula stage (data not shown). At prism stage, *Lvsnail* mRNA reappears in PMCs and becomes localized to the two ventrolateral PMC clusters (Fig. 2F,G). The expression continues at these two sites, corresponding to the tips of the arm rods of the pluteus larva (Fig. 2H). The SMC expression pattern of *Lvsnail* is consistent with the observations from a recent publication (Hardin and Illingworth, 2006).

LvSnail is required for PMC ingression

To determine the function of LvSnail in sea urchin development, we designed and injected morpholino antisense oligonucleotides (SnaMASO) into fertilized eggs to block the endogenous LvSnail translation. To test the specificity of the morpholino used here, we performed an MASO-resistant mRNA rescue experiment, and successfully rescued about 40% of the embryos (see Fig. S1 in the supplementary material); moreover, a second morpholino also exhibited the same phenotype as the morpholino reported here, though with a lower knockdown efficiency.

SnaMASO-injected embryos (‘Sna morphants’) developed normally through the cleavage and the blastula stages, and they hatched at the same time as controls. However, when PMCs of control embryos ingressed into the blastocoele (Fig. 3A, arrow), PMCs failed to ingress in Sna morphants (>90%, Fig. 3D). The PMC ingression block continued (Fig. 3F,G; 35%, 65%, respectively) even as control siblings completed gastrulation (Fig. 3C). Although in a significantly delayed fashion, almost all Sna morphants eventually displayed a normal archenteron with no apparent phenotypic defects (see Fig. S2 in the supplementary material). Other later phenotypes were observed in these Sna morphants, including loss of pigment cells, and stunted arm rod growth (see Fig. S2 in the supplementary material), but in this study we focus only on the PMC ingression phenotype.

At ingression LvSnail functions autonomously in micromeres

Chimeric embryos were generated to localize required Snail activity. To block LvSnail function specifically in the vegetal half, we combined a control animal half embryo (with FITC, shown in green) with a SnaMASO-injected vegetal half (with rhodamine-conjugated

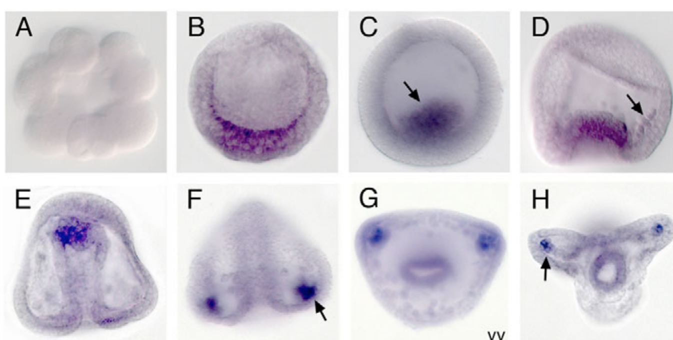

Fig. 2. WMISH showing the dynamic pattern of *Lvsnail* mRNA expression during sea urchin development. (**A**) No staining is detected at the 16-cell stage. (**B**) At late-hatched blastula, *Lvsnail* staining appears in the vegetal region. (**C**) In a mesenchyme blastula, undergoing ingression, *Lvsnail* mRNA is detected in the ingressing PMCs (arrow). (**D**) In early gastrula, *Lvsnail* mRNA expression disappears from PMCs (arrow), and is expressed instead in SMCs. (**E**) Late gastrula, *Lvsnail* mRNA continues to be expressed in SMCs. (**F**,**G**) Lateral and vegetal (vv) views, respectively, of Prism stage embryos, showing *Lvsnail* mRNA in ventrolateral PMC clusters (arrow in F). (**H**) Early pluteus. *Lvsnail* mRNA is detected in the PMCs at the tips of the larval arms (arrow).

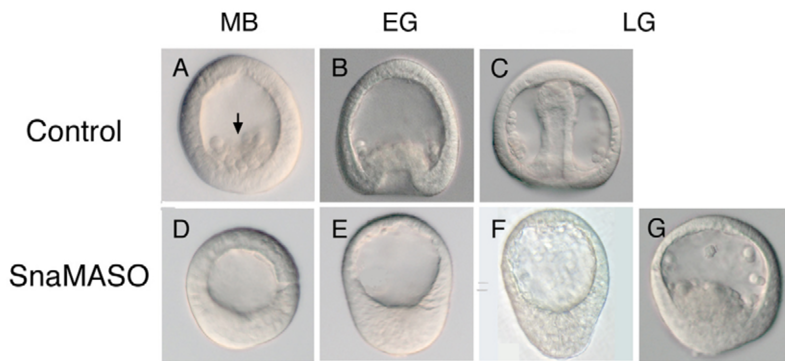

Fig. 3. PMC ingression is blocked by SnaMASO injection. (**A-C**) Control embryos show normal PMC ingression (arrow), and normal gastrulation. (**D-G**) Embryos of the same age as A-C injected with SnaMASO. Compared with the control, SnaMASO-injected embryos (Sna morphants) show no PMC ingression (D), even at gastrula stages (E-G). (G) Invagination is also delayed, though occurs normally later. MB, mesenchyme blastula; EG, early gastrula; LG, late gastrula.

dextran, shown in red) (Fig. 4G). The resulting embryos showed no PMC ingression (Fig. 4C,C′; 3/4, i.e. 3 out of 4 chimera embryos exhibited the phenotype shown in the figure), similar to Sna morphants (Fig. 4B), whereas the reciprocal chimeric embryos (SnaMASO in animal half) developed normally (Fig. 4D,D′; 4/4) compared with glycerol-injected controls (Fig. 4A). As *Lvsnail* mRNA is expressed in ingressing PMCs, which are derived from the micromere lineage, we then replaced a single micromere from a green-dyed control host with a SnaMASO-injected micromere (in red) (Fig. 4H). The red micromere progeny failed to ingress (Fig. 4E′, arrow), whereas the green micromere progeny (serving as internal controls) ingressed normally and settled at the bottom of the blastocoele (Fig. 4E,E′; arrowheads) (n=18; 5/5, 3/6, 5/7). The reciprocal experiment showed that progeny of a single green control micromere ingressed normally even when put onto a SnaMASO-injected host (Fig. 4F,F′; arrow). Taken together, these data show that Snail is required in micromeres for these cells to ingress as PMCs.

LvSnail functions downstream of early micromere specification but upstream of PMC differentiation

To gain a molecular understanding of the function of Snail in PMC ingression, we examined the expression of genes of the micromere GRN in Sna morphants (Fig. 5). Sna morphants show a significantly reduced staining of 1d5, a monoclonal antibody recognizing a PMC-specific cell surface MSP130 glycoprotein, when compared with control embryos at MB stage (Fig. 5E,F). The mRNA expression level of *Lvsm30*, *Lvsm50* and *Lvmsp130* was examined by QPCR, and all of them were significantly reduced in Sna morphants, when compared with MB controls. At the HB stage *Lvmsp130* expression showed a drastic decrease but *Lvsm50* did not (Fig. 5G). These data suggest that LvSnail acts upstream of PMC skeletogenic differentiation, but this interaction could be direct or indirect.

Two transcription factors in the micromere GRN, *alx1* (Ettensohn et al., 2003) and *ets1* (Kurokawa et al., 1999), are known to be essential for specifying early micromeres. We examined the mRNA expression of these genes in the presence of SnaMASO by WMISH and QPCR. As shown by WMISH (Fig. 5A-D) and corroborated by QPCR (data not shown), the expression level of both genes did not change in Sna morphants. Moreover, they continued to be expressed in the PMC precursors in the central region of the vegetal plate (Fig. 5B,D), even though in the absence of Snail, the PMCs failed to ingress (Fig. 5A,C). These data suggest that the PMCs are correctly specified at least to the level of these transcription factors, and further suggest that *snail* may function downstream of *alx1* and *ets1*. This hypothesis was tested next.

The mRNA expression level of *Lvsnail* was measured in Alx1 morphants and in embryos treated with a MEK inhibitor, U0126, which disrupts the ERK signaling pathway and abolishes the activity of the Ets1 protein (Rottinger et al., 2004). In Alx1 morphants, embryos showed no PMC ingression, and exhibited reduced mRNA expression of *Lvsnail* as shown by WMISH (Fig. 6A,B). In U0126-treated embryos, however, the expression of *Lvsnail* appeared to be normal regardless of the block to PMC ingression (Fig. 6C,D). Each of these results was corroborated by QPCR (Table 1). Furthermore, *Lvsnail* expression was not affected by injecting a dominant-negative form of Ets1 as measured by QPCR (Table 1). These data indicate that *Lvalx1*, but not *Lvets1*, is an upstream regulator of *Lvsnail* expression in the micromere GRN.

To directly test the hypothesis that *Lvsnail* acts downstream of *Lvalx1*, we asked if expression of LvSnail could rescue the Alx1-depleted embryos. Two separate experiments were performed. First, whole embryos were double-injected with AlxMASO and *Lvsnail* mRNA. PMC ingression is blocked by AlxMASO injection alone (Fig. 7B), and overexpression of LvSnail alone results in an excess number of mesenchyme cells in the blastocoele (Fig. 7, compare C with A). The PMC (1d5) staining shows that these extra mesenchyme cells, although highly variable, are a mixed population of PMCs and SMCs (data not shown). In AlxMASO/Snail co-injected embryos, mesenchyme cells are clearly seen in the blastocoele (n=50, Fig. 7D), supporting the hypothesis that *snail* functions downstream of *alx1*. Second, to confirm that the rescue is autonomous to the micromeres, a chimeric experiment asked if LvSnail rescues PMC ingression in the absence of LvAlx1 protein in micromeres. We generated chimeric embryos by replacing two micromeres in control hosts. One micromere contained AlxMASO (dyed red) inserted to replace one host micromere, and a second micromere was either from an FITC-injected control embryo (to serve as an experimental control) or from an AlxMASO/Snail co-injected embryo (dyed green) (Fig. 7E). Most red micromeres (AlxMASO injected) failed to ingress and stayed at the tip of the archenteron (ingression percentage: 1/6; 5/27), in two sets of experiments (Fig. 7F-G). In controls, all green micromeres ingressed into the blastocoele (6/6) (Fig. 7F-F″). In the double-injected experimental set, more than half of the AlxMASO/Snail co-injected

Table 1. Effects of different perturbations on the expression level of Snail as measured by QPCR

Perturbation	Snail (ΔC_t)
U0126-treated	NS/ NS, NS
DN-Ets1 MOE	NS
AlxMASO	–2.54/–3.4, NS

Data listed are considered significant, whereas non-significant effects (normalized CT difference from control is greater than –1.6 or less than +1.6) are shown as NS. Commas separate replicate measurements in the same cDNA batch; the solidus separates different batches of cDNA from independent experiments.

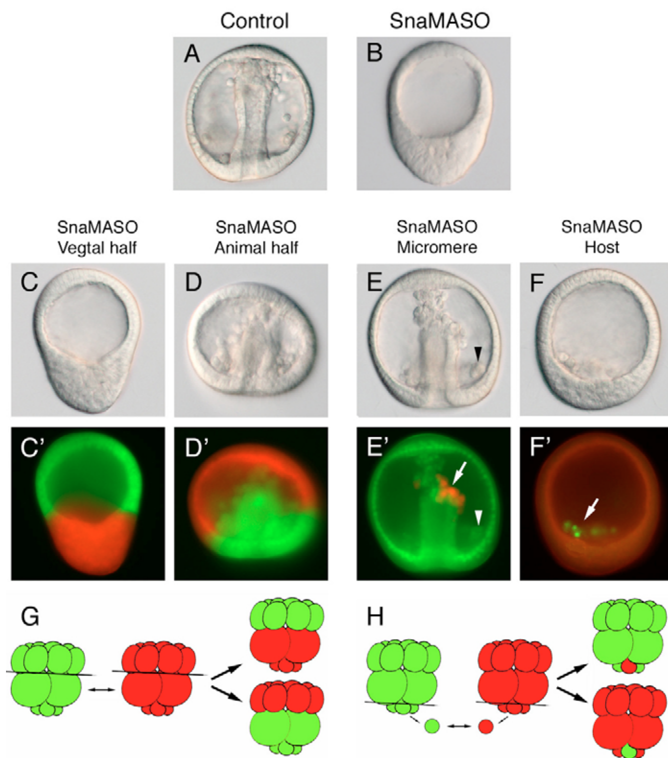

Fig. 4. Chimeric embryos demonstrate Snail is required in micromeres for ingression. (**A**) Control embryo. (**B**) SnaMASO-injected embryo showing no PMC ingression. (**C**,**C′**) A SnaMASO-containing vegetal half (red) was combined with a control animal half (green). Resulting embryos lack PMCs. (**D**,**D′**) A SnaMASO-containing animal half (red) was combined with a control vegetal half (green). Resulting embryos develop PMCs as in sibling controls. (**G**) A schematic diagram of the experimental designs of C and D. (**E**,**E′**) Single SnaMASO-containing micromere (red) transplanted onto a control host embryo lacking one micromere (green). The SnaMASO micromere failed to ingress (arrow in E′), whereas all other control micromeres ingressed and migrated normally (arrowheads). (**F**,**F′**) The reciprocal experiment to that in E. One normal micromere (green) ingresses into the blastocoele (arrow in F′) when transplanted to a SnaMASO-injected host embryo lacking one micromere (red). (**H**) The schematic diagram of the experimental designs of E and F. See text for details.

green micromeres ingressed (15/27) (Fig. 7G-G″). From these chimeric experiments, this result suggests that Snail is sufficient to rescue the ingression of AlxMASO-injected micromeres at least partially, and is consistent with the notion that *snail* functions downstream of *alx1* in the micromere-PMC GRN.

LvSnail downregulates cadherin expression as a component of its function

Cadherin is expressed in every cell of the embryo beginning early in cleavage. At ingression, PMCs rapidly lose cadherin from their cell surfaces. In other model systems, it has been documented that Snail represses the transcription of cadherin. To approach this question in sea urchins, we used an early transcription factor, *pmar1*, as an experimental tool. *pmar1* is upstream of both *alx1* and *ets1*, and is necessary for early initiation of the micromere specification program (Oliveri et al., 2002; Oliveri et al., 2003). Overexpression of Pmar1 transforms most cells of the embryo into PMCs (Oliveri et al., 2002). Therefore, it was hypothesized that SnaMASO would block this Pmar1 ectopic-expression phenotype, if, in fact, Snail is required for EMT. Accordingly, *Lvpmar1* was cloned and injected into eggs. Compared with a normal control (Fig. 8A), most cells of the *Lvpmar1*-injected embryo were converted into PMCs when Pmar1 was ectopically expressed (Fig. 8B), as expected from Oliveri et al. (Oliveri et al., 2002). In Pmar1-overexpressing embryos, we next measured the mRNA expression of *Lvsnail*, *Lvalx1* and *Lvmsp130* by QPCR, and as expected, the expression level of all three genes was upregulated (Fig. 8D). WMISH of *Lvsnail* showed a ubiquitous expression, also as might

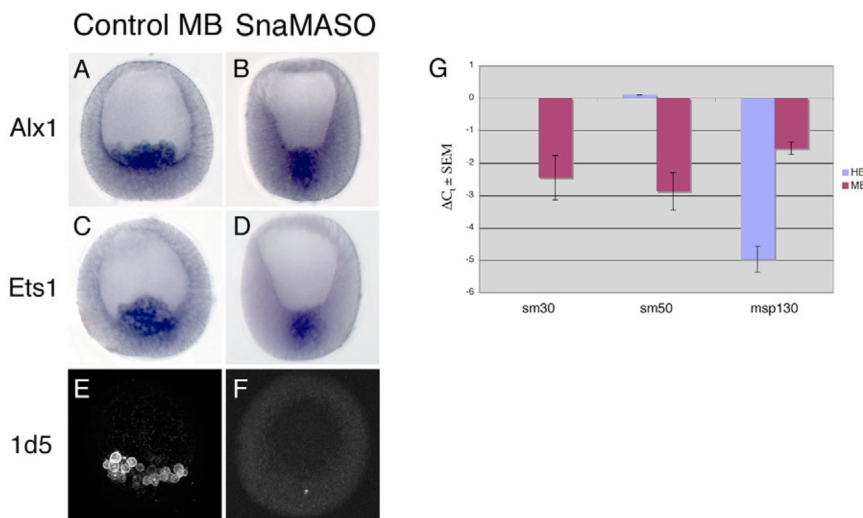

Fig. 5. Effects of SnaMASO on PMC specification and differentiation. (**A-D**) In situ hybridization with *Lvalx1* and *Lvets1* probes. Control mesenchyme blastula embryos show strong expression of *Lvalx1* and *Lvets1* in PMCs (A,C). Expression of *alx1* and *ets1* are not affected by SnaMASO injection (B,D). (**E**,**F**) Immunostaining with mAb 1d5, shows the presence of 1d5 in control PMCs (E), but little to no expression of 1d5 in Sna morphants (F). (**G**) QPCR analysis of the expression of PMC differentiation genes, *Lvsm30*, *Lvsm50* and *Lvmsp130*, in Sna morphants at the HB and MB stage relative to controls. Data are shown as net $\Delta C_T \pm$ s.e.m.

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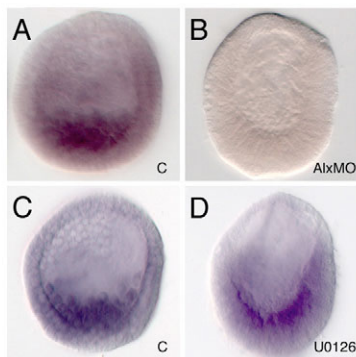

Fig. 6. Upstream regulators of LvSnail. (**A**,**B**) WMISH shows a significant reduction of *Lvsnail* mRNA expression in Alx1 morphants (B), compared with control embryos (A). (**C**,**D**) Effect of U0126 treatment on *Lvsnail* mRNA expression. The U0126-treated embryos show no significant changes in Snail expression (D) compared with controls (C).

be expected for a gene downstream of *pmar1* (data not shown) and consistent with the Oliveri et al. conclusion that most or all cells were converted into PMCs.

When embryos were co-injected with Pmar1 and SnaMASO, a striking difference was observed. About 60-70% of the co-injected embryos showed no ingression at all (*n*=200, Fig. 8C), and the remaining embryos showed minimal ingression (data not shown). None of the co-injected embryos exhibited a complete mesenchymal transformation or lost the epithelial integrity like those injected with Pmar1 alone (Fig. 8B). Thus, ectopic expression of Pmar1 requires Snail expression to complete the transformation and ingression of ectopic cells in the embryo.

Because many studies in cell culture of tumor cells and in mammals showed evidence that Snail controls EMT in part by repressing E-cadherin expression (Batlle et al., 2000; Cano et al., 2000; Carver et al., 2001), we next asked if a similar repression or downregulation occurs in sea urchins. First, although Snail family genes act as transcription repressors, to confirm that Snail functions as a repressor in sea urchin, we converted LvSnail into an obligate repressor by combining the Engrailed repressor domain with the DNA-binding domain of LvSnail protein, and injected the mRNA of this fusion construct into fertilized eggs. As expected, the observed phenotypes were the same as seen in wild-type LvSnail-injected embryos (Fig. 7C; data not shown). These data support the hypothesis that LvSnail normally functions as a repressor in the embryo. Next, to examine whether LvSnail downregulates the expression of Cadherin, we measured and compared the mRNA expression of LvG-cadherin in Pmar1-injected embryos and Pmar1/SnaMASO co-injected embryos by QPCR. It was necessary to use Pmar1 as a tool because LvG-cadherin expression is ubiquitous in the embryo, and a detectable change was not observed by QPCR in Sna morphants, where PMCs constitute only 5% of the cells. In Pmar1-injected embryos, the expression level of LvG-cadherin showed a significant decrease by QPCR (a signature of mesenchyme cells), whereas an increase of the LvG-cadherin expression was observed in embryos co-injected with Pmar1 and SnaMASO, which is also consistent with the rescued phenotype (Fig. 8C). This experiment demonstrates, therefore, that Snail downregulates the expression of Cadherin at PMC ingression in sea urchin embryos.

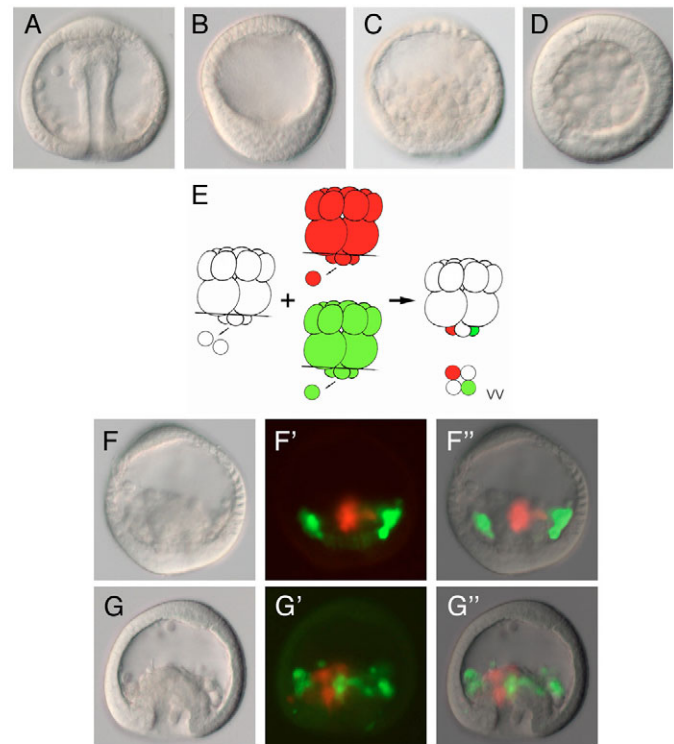

Fig. 7. LvSnail functions downstream of LvAlx1 in PMCs. (**A**) Control embryos. (**B**) Embryos injected with AlxMASO show no PMC ingression. (**C**) Excess mesenchyme cells form in embryos overexpressing LvSnail. (**D**) Co-injection of LvSnail rescues the formation of mesenchyme cells in the absence of LvAlx1. (**E**) Diagram of experiment in F,G. vv, vegetal view. (**F-F″**) Chimeric embryos generated by combining one control micromere (green) and one AlxMASO-containing micromere (red) with a control 16-cell stage embryo in which two micromeres were removed. Brightfield (F), fluorescent (F′) and merged images (F″) show that the AlxMASO-containing micromere progeny do not ingress, unlike the control micromere progeny in the same embryos. (**G-G″**) LvSnail can rescue the effects of AlxMASO in PMC ingression. The micromere co-injected with *Lvsnail* mRNA and AlxMASO (green) ingresses into the blastocoele, whereas the AlxMASO-injected micromere (red) fails to ingress. Brightfield (G), fluorescent (G′), and merged (G″) images are shown.

LvSnail positively regulates cadherin endocytosis in PMCs

At PMC ingression, the junction-associated cadherin-catenin complex is completely endocytosed (Miller and McClay, 1997a; Miller and McClay, 1997b) and removed from the cell membrane of mesenchyme cells. As the absence of LvSnail severely blocks PMC ingression, it is possible that LvSnail may play a role in regulating that endocytosis process. To test this hypothesis, a GFP reporter construct (CadTM-GFP) was used. CadTM-GFP recapitulates the apical localization of the cadherin complex when expressed (Fig. 8E) and also forms punctate foci of the intracellular GFP signal when endocytosed in ingressing PMCs (arrowheads in Fig. 8F,G). Using this construct the following experiment was performed as shown in Fig. 8H. Pmar1 and CadTM-GFP were co-injected into the eggs and followed by injection at the two-cell stage with SnaMASO (with rhodamine-dextran, shown in red) into only one blastomere (also see Fig. 8H). In about 60% of SnaMASO two-cell injection embryos (*n*=70), SnaMASO successfully blocked PMC ingression

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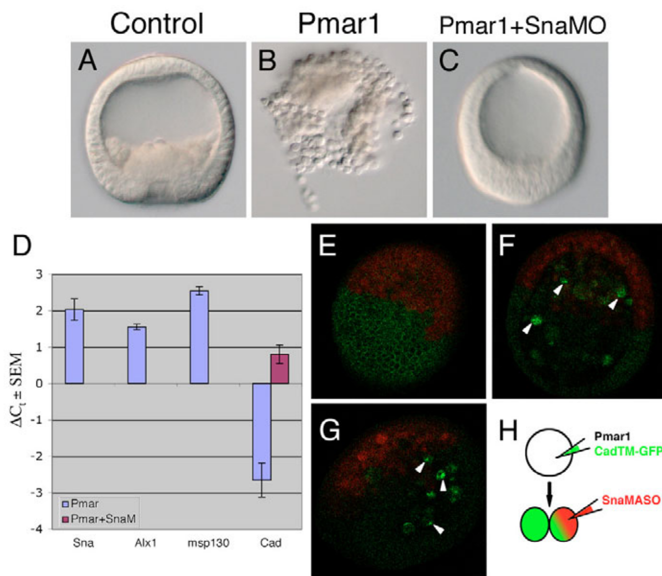

Fig. 8. LvSnail downregulates G-cadherin expression and positively regulates its endocytosis. (**A**) Control embryos. (**B**) Embryos with ectopically expressed Pmar1 produce extra PMCs and mesenchyme extrusions. (**C**) Co-injection of SnaMASO rescues the Pmar1 ectopic-expression phenotype. Most co-injected embryos fail to undergo PMC ingression. (**D**) QPCR analysis of Pmar1- and Pmar1/SnaMASO-injected embryos. The expression of *Lvsnail*, *Lvalx1*, and *Lvmsp130* is upregulated in Pmar1-injected embryos, whereas G-cadherin is significantly downregulated. The co-injection of SnaMASO rescues the reduction of cadherin expression in Pmar1-overexpressed embryos. Data are shown as net ΔC_T±s.e.m. (**E**) Surface view of an embryo injected at the one- and two-cell stage as illustrated in H. The SnaMASO-injected half is shown in red. The apical localization of cadherin can be clearly observed in adherens junctions (CadTM-GFP; adherens junctions are present on the red side also but obscured in the dual image by the red dye). (**F**) The internal view of the same embryo as shown in E; the intracellular punctate GFP signals indicate endocytosed cadherins (arrowheads), which do not overlap with SnaMASO-injected cells (with rhodamine-dextran). (**G**) Another two-cell injection embryo; the punctate GFP signals (arrowheads) can be seen in PMCs. (**H**) Schematic of the experimental design in E-G.

in the injected half, and most ingressing PMCs came from the other half, where no SnaMASO was present; injecting rhodamine-dextran only had no effect (data not shown). Examination of mesenchyme cells in these embryos shows almost no punctate intracellular GFP to be present in the rhodamine-marked cells (those containing SnaMASO); punctuate intracellular GFP is also largely excluded from those few rhodamine-marked cells that do ingress (Fig. 8F,G; two different embryos), whereas punctate endocytic vesicles were present in all ingressed cells expressing the CadTM-GFP construct alone. These results indicate that SnaMASO impairs the endocytosis of cadherin. Therefore, Snail does indeed positively regulate the process of cadherin endocytosis, although the exact mechanism is still not understood.

DISCUSSION

EMTs initiate morphogenetic movements of many embryos including all mesoderm, the neural crest, heart, musculoskeletal system, craniofacial structures and peripheral nervous system of vertebrates (Thiery, 2002). In sea urchin embryos, EMTs occur at PMC ingression and during the formation of some SMCs. In this study, we have characterized a Snail gene in the sea urchin embryo, and show that Snail is required for PMC ingression, which is consistent with Snail being an evolutionarily conserved modulator of cell movements, rather than determining cell fates (Barrallo-Gimeno and Nieto, 2005). Moreover, with the micromere GRN, perturbation studies place Snail as a link between specification and morphogenesis of PMCs. Snail is expressed late in specification of micromeres, just before ingression is launched.

Sea urchin Snail function is required for PMC ingression

The conclusion that Snail is required for PMC ingression is supported by several independent observations. First, functional knockdown of Snail with SnaMASO blocks the first event in PMC morphogenesis (EMT/ingression) (Fig. 3), as well as the expression of several PMC differentiation genes (Fig. 5G). Second, the SnaMASO chimeric experiments demonstrate that Snail function is necessary in micromeres for ingression to occur (Fig. 4), as Snail-deficient micromere descendants do not migrate into the blastocoele of a normal host embryo, and stay at the tip of the archenteron (Fig. 4E,E′); absence of Snail everywhere else in the embryo has no effect on the Snail-expressing micromere, which retains its ability to ingress (Fig. 4F,F′). Taken together, these data clearly indicate that Snail is involved in control of the EMT process, particularly during PMC ingression.

Although Snail family genes are known to be involved in EMTs in metastatic progression of tumors, mesoderm development, and neural crest cell migration in vertebrates (for reviews, see Barrallo-Gimeno and Nieto, 2005; Hemavathy et al., 2000; Nieto, 2002), our finding here is nonetheless intriguing as this is the first report to date showing that an invertebrate Snail gene controls a bona fide EMT (i.e. PMC ingression), which is characterized by cells migrating as individual cells through the extracellular matrix. Snail also has been shown to be involved in cell movements that do not require a full EMT in different experimental models. During mesoderm formation in *Xenopus* embryos, for example, individual mesenchymal cells are not formed, but rather a mass of sheet-like epiblast cells penetrate the blastocoele (i.e. involution), during which cells maintain contact with each other while migrating (Keller et al., 2000). Likewise, during gastrulation in *Drosophila*, the cells of the invaginating ventral furrow give rise to mesoderm. This migration of the presumptive mesoderm occurs as a group of cells, where cell-cell adhesion is reduced but maintained due to a switch in expression from E- to N-Cadherin (Oda et al., 1998). Thus, the cell population remains adherent enough to move as an intact sheet. Other similar processes can be observed in hair bud formation (Jorda et al., 2005), or wound healing (Savagner et al., 2005) in mice. Hence, Snail genes are involved not only in full EMTs in most deuterostome lineages, but also in various types of cell movements throughout the animal kingdom (Barrallo-Gimeno and Nieto, 2005; De Craene et al., 2005b).

Downregulation of cadherin expression by Snail is conserved in sea urchins

Drosophila and mouse Snail mutants fail to downregulate the expression of the cell adhesion molecule E-cadherin (Carver et al., 2001; Oda et al., 1998) during gastrulation, and, in mammalian epithelial cells, Snail protein has been shown to bind to the promoter region of *E-cadherin*, and repress its transcription (Batlle et al., 2000; Cano et al., 2000). In sea urchin Sna

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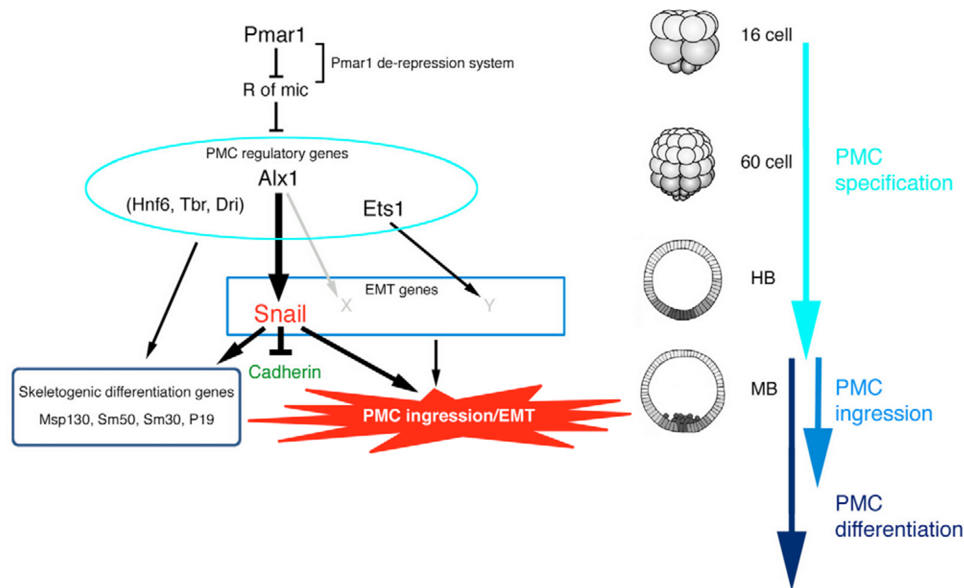

Fig. 9. Model of a Snail-dependent pathway regulating PMC ingression in the PMC-micromere GRN. Pmar1 de-repression system initiates the entire PMC specification program, and activates Alx1, Ets1 and other PMC regulatory genes (light blue oval), which in turn regulate skeletogenic differentiation genes (dark blue box). Alx1 regulates Snail (and other unknown EMT genes, denoted as X), and Snail represses Cadherin to attenuate the cell-cell adhesion, which allows PMCs to ingress into the blastocoele. Ets1 also regulates PMC ingression via unidentified EMT genes (denoted as Y). A subnetwork of EMT genes (light blue square box) regulates the EMT process of PMCs. The developmental stages shown on the left correspond to the chronological sequences of PMC developmental processes shown on the right.

morphants, a failure to downregulate cadherin expression occurs and this, in part, may explain the inability of PMCs to ingress when Snail is eliminated. This hypothesis is strongly supported by the outcome from the Pmar1/SnaMASO co-injection rescue experiment (Fig. 8), which allowed us to examine the repression (directly or indirectly) of cadherin by Snail in vivo, under conditions where all or most cells of the embryo were converted to micromeres. Thus, the downregulation (or repression) of cadherin expression by Snail in association with cell movement appears to be well conserved in insects, sea urchins and vertebrates (this study) (Oda et al., 1998; Carver et al., 2001; Jamora et al., 2005; Yamashita et al., 2004). Indeed, E-cadherin loss of expression leads to tumor progression (Perl et al., 1998), and the transcriptional repression of cadherin expression by Snail plays a major role in the EMTs (Batlle et al., 2000). However, cadherins can be functionally inactivated by other different mechanisms, such as post-translational control (Cavallaro and Christofori, 2004). Moreover, a recent publication has shown that p38 downregulates E-cadherin post-transcriptionally during mouse gastrulation (Zohn et al., 2006), although in sea urchin, loss of p38 activity affects neither the ingression of PMCs nor SMCs (Bradham and McClay, 2006).

Cadherins can also be rapidly removed from cell membranes by endocytosis and/or degradation (reviewed by D'Souza-Schorey, 2005; Lu et al., 2003; Janda et al., 2006). In the sea urchin embryo, cadherin endocytosis occurs during PMC ingression (Miller and McClay, 1997a), and this appears to be positively regulated by Snail (as shown in Fig. 8E-G). Thus, Snail regulates cadherin removal from cell membranes via endocytosis, and it also regulates termination of cadherin transcription, both of which enable the transformation and maintenance of the mesenchyme cell phenotype. Snail therefore orchestrates a double mechanism to eliminate cadherin during the EMT process.

Lvsnail is expressed not only in PMCs but in other mesodermal cell populations as well. Some secondary mesenchyme cells (e.g. pigment cells and blastocoelear cells) also undergo a similar EMT process prior to their migratory behaviors. Preliminary data from SnaMASO perturbation studies suggest that Snail is involved in those EMTs as well (see Fig. S3 in the supplementary material). Thus, it is highly possible that Snail plays an essential role upstream of each EMT event during sea urchin embryogenesis. In addition Snail might also be involved in other aspects of development, given its dynamic expression pattern. These potential functions were not explored here, but warrant further analyses.

Snail acts downstream of Pmar1 and Alx1 in the micromere-PMC GRN

Our results show that Pmar1 and Alx1 are positive regulators of Snail mRNA expression, and the ability of both transcription factors to influence PMC ingression operates through Snail (see Fig. 9).

Pmar1 responds to the maternal β-catenin signal and initiates the entire PMC GRN (Oliveri et al., 2003). Overexpression of Pmar1 significantly elevates the expression level of Snail (Fig. 8D), as expected if Pmar1 is upstream of Snail. Snail is also downstream of Alx1, an important PMC specifier, known to be under control of the Pmar1 de-repression system (Ettensohn et al., 2003). In the absence of Alx1, Snail expression decreases significantly (Fig. 6A,B), whereas Alx1 expression is unaffected in Sna morphants (Fig. 5A,B). Further, Snail expression rescues Alx1 knockdown, at least partially. These results strongly support the notion that Snail acts downstream of micromere specification, but upstream of, and is required for, PMC ingression. Snail mRNA injection rescues the ingression of Alx1-depeleted micromeres in about 60% of the chimera embryos (Fig. 7). This partial rescue suggests that Alx1 regulates PMC ingression partly

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through Snail but may require other unknown gene targets, thereby preventing a full rescue phenotype. Nevertheless, the rescue observed here further validates the designated position of Snail in the PMC GRN (Fig. 9).

Ets1 has been shown to impact PMC ingression when perturbed (Kurokawa et al., 1999; Rottinger et al., 2004). Our data show that Snail is neither upstream nor downstream of Ets1. Ets1 mRNA expression is unaffected in the absence of Snail (Fig. 5C,D), and Snail mRNA expression is also unaltered in U0126-treated embryos (Fig. 6C,D). These results lead us to conclude that, even though Ets1 is an important upstream regulator of PMC formation, the effect of Ets1 on PMC ingression does not function through Snail, but likely through other unidentified factors, which are also involved in the EMT process of PMCs (denoted as Y in Fig. 9).

There are several additional transcription factors already identified in the PMC GRN, including *dri* (Amore et al., 2003), *hnf6* (Otim et al., 2004) and *tbr* (Croce et al., 2001; Fuchikami et al., 2002). Although these genes are expressed earlier than Snail, they cannot be upstream regulators of Snail, as perturbation of these genes fails to show any effect on PMC ingression. Instead these transcription factors primarily affect the skeletogenic differentiation of PMCs. It is clear that many of the PMC components are specified in a pathway that is independent of Snail expression so it is not surprising that a substantial number of micromere transcription factors operate independently of Snail and do not require Snail for their function.

Three PMC terminal differentiation genes were examined in this study. Our data show that Snail positively influences *sm30*, *sm50* and *msp130* expression. Given the fact that Snail itself is a transcriptional repressor, Snail must indirectly regulate these genes by repressing an (or some) unknown repressor(s), a regulatory device that is similar to the Pmar1 de-repression system. The initiation of *sm30* expression may be relatively proximal to Snail function, as *sm30* transcripts accumulate immediately after PMC ingression (Guss and Ettensohn, 1997), and Snail is expressed in PMC clusters (Fig. 2F,G), where *sm30* is also highly expressed later at prism stage (Guss and Ettensohn, 1997). On the other hand, both *sm50* and *msp130* expression are initiated earlier than, and therefore independently of, Snail expression (Guss and Ettensohn, 1997) (data not shown). Thus it is likely that Snail regulates the maintenance of expression of these genes in PMCs at ingression. Further identification of Snail target genes will help elucidate relationships between Snail and the PMC differentiation program, and also help unravel the connection between pre- and post-EMT gene network states in PMCs.

A subnetwork of EMT genes controls PMC ingression

The Pmar1>Alx1>Snail>Cadherin hierarchical regulatory relationship we show here reveals one trajectory through the micromere GRN. That trajectory is necessary for ingression, but it is not the exclusive pathway required, as evidenced by the Ets1 data and by the incomplete rescue of Alx1 morphants. Even so, the functional analyses of Snail and perturbation studies establish a tight link between early micromere specification and PMC ingression (Fig. 9). Previous studies of several PMC regulatory genes in the GRN, including *pmar1*, *alx1* and *ets1*, all focused on their functions on specifying micromeres in advance of the differentiation of PMCs. Here, we show that in addition to preparation for differentiation, the specification through Snail enables the cells to transit into morphogenesis. Other transcription factors govern various mechanisms of differentiation independently of the pathway through Snail.

With Snail occupying an important role in regulating PMC ingression, this study provides groundwork for investigating the molecular basis of EMT in PMCs, and further strengthens the hypothesis that a group of genes controls the EMT of PMCs in the sea urchin embryo [as previously suggested in Fernandez-Serra et al. (Fernandez-Serra et al., 2004)]. We propose that this subnetwork of EMT genes (including *snail*), which functions downstream of the micromere-PMC specification program, attenuates cell-cell adhesion (Fink and McClay, 1985; Hertzler and McClay, 1999), and upregulates molecules associated with cell motility changes, such as Rho GTPases (Liu and Jessell, 1998) and metalloproteinases (MMPs) (Yokoyama et al., 2003; Miyoshi et al., 2004; Jorda et al., 2005; Ingersoll and Pendharkar, 2005). Eventually, this complex subnetwork orchestrates an EMT event by summing up the spectrum of molecular and cellular changes, and then triggers PMC ingression.

Future investigations both in PMC formation in the sea urchin and in comparative studies, will be of value to further understand how other transcription factors operate with Snail to engage the EMT mechanism. Thus, construction of a more complete pre-EMT subnetwork will indeed contribute to the understanding of the mechanism(s) controlling PMC ingression in the sea urchin, and also provide useful insight into the complex molecular strategies that regulate EMT events in other organisms.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/6/1061/DC1

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