GENE REGULATORY NETWORKS CONTROLLING AN
EPITHELIAL-MESENCHYMAL TRANSITION

by

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Date: April 19, 2007

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT
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Abstract

Epithelial-mesenchymal transitions (EMTs) are fundamental and indispensable to embryonic morphogenesis throughout the animal kingdom. At the onset of gastrulation in the sea urchin embryo, micromere-derived primary mesenchyme cells (PMCs) undergo an EMT process to ingress into the blastocoel, and these cells later become the larval skeleton. Much has been learned about PMC specification in sea urchin embryos. However, much less is known about how states of the sequentially progressing PMC gene regulatory network (GRN) controls the EMT process during PMC ingression. Transcriptional regulators such as Snail and Twist have emerged as important molecules for controlling EMTs in many model systems. Sea urchin snail and twist genes were cloned from Lytechinus variegates, and each has been experimentally connected to the PMC regulatory network; these experiments demonstrate several requirements for PMC ingression, and in doing so, begin to illustrate how a gene regulatory network state controls morphogenesis.

Functional knockdown analyses of Snail with morpholino-substituted antisense oligonucleotides (MASO) in whole embryos and chimeras demonstrated that Snail is required in micromeres for PMC ingression. Investigations also show that Snail down-regulates cadherin expression as an evolutionarily conserved mechanism, and Snail positively regulates a required endocytic clearance of epithelial membrane molecules during EMT. Perturbation experiments indicate that Twist has accessory roles in regulating PMC ingression, and possibly plays a maintenance role in PMC specification network state. In addition, Twist also functions in the post-EMT network state, particularly in directing PMC differentiation and skeletogenesis.

The recently annotated sea urchin genome accelerates the discovery of new genes and holds strong promise of mapping out a complete canvas of the micromere-PMC gene
regulatory network. Using the genome resources we successfully cloned several newly identified PMC genes, and found most of them to be expressed in micromeres just prior to ingression of the nascent PMCs. Current experiments focus on the roles of these genes in preparing for, executing, and/or controlling the mesenchymal behavior following PMC ingression. The functions and inter-relationships of these genes will greatly augment our understanding of how a gene regulatory network state controls a crucial morphogenetic event.
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they can be proud of themselves for raising a son who becomes who I am today. I forever thank you, Mom and Dad. You guys are the best!
Dedication

This dissertation is dedicated to

Yu-Ping Yang, who is the love of my life,

and to my parents

Mei-Hsiow Wu-Lee and Shou-Fu Wu, who are always supporting me.
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List of Abbreviations

EMT: Epithelial-mesenchymal transition
GRN: Gene regulatory network
MASO: Morpholino-substituted antisense oligonucleotide
PMC: Primary mesenchyme cell
QPCR: Quantitative PCR (polymerase chain reaction)
RACE: Rapid amplification of cDNA ends
RT-PCR: Reverse transcriptase PCR
SMC: Secondary mesenchyme cell
WMISH: Whole mount in situ hybridization
Chapter 1:

Introduction
Gastrulation employs many remarkable molecular and cellular mechanisms that organize the basic body plan of all bilaterians in a highly coordinated fashion. After the embryonic germ layers are specified, the cells embark on a series of morphogenetic movements that encompass the central feature of gastrulation. One of many such movements is the “epithelial-mesenchymal transition (EMT)”, which occurs multiple times during critical phases of embryonic development, and is characterized by epithelial cells converting into mesenchymal cells during this process.

The inter-conversion between the epithelial and mesenchymal states of cells was first described by Frank Lillie in the early 1900s (Lillie, 1908). In the next several decades, more examples of this “epithelial-mesenchymal transition” were observed and greatly described (e.g. Gustafson and Wolpert 1967; Solursh et al., 1978; Tosney et al., 1978; (Fink and McClay 1985), and was also recognized as a distinct cellular process (Greenburg and Hay 1982). However, the EMTs only became more amenable to experimentation after the discovery, by Michael Stoker and Michael Perryman, that Madin–Darby canine kidney (MDCK) cells could be converted from their original polarized epithelial states into migratory fibroblasts by incubation with conditioned medium from cultured fibroblasts (Stoker and Perryman 1985). The “factor” involved in this remarkable transition was later identified as hepatocyte growth factor (Nakamura et al. 1989), but at that time these experiments opened a new chapter for techniques to analyze EMTs with revolutionary molecular tools. After numerous studies and years, the mechanisms that govern EMT are just now starting to be understood at the causal molecular level, and many parallels are being found between this process in embryonic development, in tissue culture transitions and in tumor metastases (reviewed in detail in (Thiery and Sleeman 2006).

This introductory chapter reviews our current understanding of the EMT mechanisms in both embryonic development and cancer metastasis, and briefly
introduces the early endomesoderm specification leading to EMT in the sea urchin embryo. The subsequent research chapters then consists of experiments and analyses that allow the construction of an EMT gene regulatory network state in the sea urchin embryo at the time of primary mesenchyme cell (PMC) ingression. The focus of this work is on investigating the functions of the genes, Snail and Twist, in regulating PMC ingestion and mesoderm formation.

**EMT and *Drosophila* Mesoderm**

Early genetic evidence and important molecules involved in EMT were identified during research into *Drosophila* gastrulation movements. Upon the completion of cellularization, a stripe of ventral cells in the fly embryo invaginates as an epithelium, and then subsequently undergoes an EMT. These newly formed mesenchymal cells migrate dorsally along the ectoderm before differentiating into visceral, somatic, and cardiac mesoderm. This ensemble of gastrulation events is the end result of a gene regulatory network, which is controlled by Dorsal (nuclear factor NF-κB) (summarized in Figure 1.1).

Dorsal is involved in the establishment of the dorso-ventral axis, and participates in the specification of mesodermal and ectodermal lineages prior to gastrulation. Its activity is responsible for the activation of Snail and Twist, both of which exhibit defects in gastrulation when mutated in presumptive mesoderm. These two genes (among others) have been shown in *Drosophila* to be absolutely crucial for the specification of mesoderm and the formation of the ventral furrow (see reviews in Leptin 1999; Leptin 2005). After being activated by Dorsal, Twist then activates one of its downstream targets, Snail, which downregulates the transcription of Shotgun (the
**Figure 1.1: A Gene Regulatory Network Controlled by Dorsal during *Drosophila* Gastrulation.** (A) Diagram showing a cross-section of a *Drosophila* embryo. The expression domains of two Dorsal target genes, Twist (dark blue line) and Snail (light blue line), are shown relative to Dorsal expression. Darker orange circles represent high levels of nuclear Dorsal, whereas lighter circles represent lower levels of nuclear Dorsal (D = dorsal; V = ventral). Darker orange circles represent high levels of nuclear Dorsal, whereas lighter circles represent lower levels of nuclear Dorsal. (B) Genetic hierarchy of representative downstream genes of Dorsal. The highest level of Dorsal, found in the prospective mesoderm, is able to activate genes such as Twist and Snail. Heartless also can respond directly to Dorsal, but in the embryo, Heartless critically depends on Twist’s activity. The transcription factor Twist acts as an activator promoting its own transcription as well as that of Snail, Fog, T48, Heartless and Dof (i.e. Downstream of FGF). Snail is a repressor that represses E-cadherin (i.e. Shotgun), and is predicted to block the expression of the Fgf8 genes in the mesoderm.
Figure 1.1: A Gene Regulatory Network Controlled by Dorsal during *Drosophila* Gastrulation
orthologue of vertebrate E-cadherin) in mesodermal cells that undergo invagination at the ventral furrow (Oda et al. 1998). Recent studies have uncovered a new transmembrane gene, T48, which is a downstream target of Twist. T48 functions together with Folded gastrulation (Fog), a secreted molecule also downstream of Twist (Costa et al. 1994), independently to, and in parallel with, Snail to regulate the adherens junctions and cytoskeletal rearrangements (Kolsch et al. 2007). More ongoing efforts are poised to find out missing pieces within this complex regulatory network controlling this elegant morphogenetic process (Stathopoulos and Levine 2004; Seher et al. 2006; Sandmann et al. 2007; Zeitlinger et al. 2007).

The invaginated mesoderm in Drosophila has to undergo an EMT before its migration along the ectoderm. FGF signaling is required for the efficient spreading of the mesoderm on the ectoderm, but not for EMT, although FGF signaling is active in the mesoderm during the EMT process (Shishido et al. 1993; Beiman et al. 1996; Gisselbrecht et al. 1996; Gryzik and Muller 2004; Stathopoulos et al. 2004). While in mouse embryo, FGF signaling is responsible for E-cadherin downregulation by maintaining Snail expression in the late primitive streak to generate mesoderm (Ciruna and Rossant 2001). In addition, Snail-/- embryos die during gastrulation and exhibit mesoderm migration defects (Carver et al. 2001).
EMT and Neural Crest

Neural crest cells, often referred as “the fourth germ layer”, undergo an EMT within the dorsal neural epithelium in vertebrates, and subsequently migrate over long distances before giving rise to many different derivatives. The neural crest territory is progressively determined in a rostrocaudal gradient along the neural axis of the vertebrate embryo at the interface (neural plate border) between the neural tube and the lateral non-neural ectoderm. This cell population is an excellent model system for the study of EMTs, even though it is a difficult system to dissect during the moment-to-moment sequence of EMT. Its strengths are the abilities to study its induction and the subsequent migration and differentiation, which are under the complex control of overlapping signaling pathways (summarized in Figure 1.2).

The neural crest territory is initiated by BMP, Wnt, Notch and FGF signaling molecules, which are secreted from the underlying mesoderm and adjacent non-neural ectoderm (Liem et al. 1995; Bonstein et al. 1998; LaBonne and Bronner-Fraser 1998; Marchant et al. 1998; Garcia-Castro et al. 2002; Monsoro-Burq et al. 2003). These intercellular signals also function simultaneously to segregate neural from non-neural ectoderm during neural induction (Sasai and De Robertis 1997; Weinstein and Hemmati-Brivanlou 1999). These signals induce a set of genes to specify a “specialized” region at the border of the neural plate (Figure 1.2A). The collective expression of many transcription factors, including Zic3, Pax3/7, Dlx3/5 and Msx1/2, defines a broad territory containing cells competent to form the “bona fide” neural crest. These so-called “neural plate border specifiers” themselves activate a suite of transcription factors (Fig. 1.2B), including Snail1, Snail2 (formerly Slug), AP2, FoxD3, Twist, Id, Sox9/10, and c-Myc, to further refine the neural crest specification, and promote an EMT, migration and later terminal differentiation (see reviews in Basch et al. 2004; Barembaum and
**Figure 1.2: Model of neural crest induction** (using data from several organisms but with chick neural crest formation illustrated). (A) Interactions between the non-neural ectoderm (green) and the mesendoderm below (not shown) with the prospective neural plate (blue) induce the neural plate border (black) to express several transcription factors (neural plate border specifiers). (B) As neural crest cells are specified progressively along with the neurulation process, the expressions of many neural crest specification genes are activated after the neural plate border (black) is specified. Paraxial mesoderm is shown in gray. (C) Near the time of neural tube closure (depending on the species), the neural crest cells go through an epithelial to mesenchymal transition and delaminate from the neural folds or dorsal neural tube (blue) and migrate along defined pathways. Somites are shown in gray. Figure adapted from Barembaum and Bronner-Fraser, 2005.
Figure 1.2: Model of neural crest induction
Bronner-Fraser 2005). Many reports demonstrate that these transcription factors cross-regulate to sustain each other’s expression in order to gradually specify and maintain the cell fate of the nascent neural crest (Sasai et al. 2001; del Barrio and Nieto 2002; Aoki et al. 2003; Aybar et al. 2003; Bellmeyer et al. 2003; Honore et al. 2003; Luo et al. 2003). Nevertheless the precise hierarchical relationships among these transcription factors are currently difficult to assign. Moreover, in different species (mouse, frog, and chick), similar inter-relationships are apparently present, although several differences have been noted perhaps resulting from species-specific deployments of these “neural crest specifiers” (Sefton et al. 1998; Locascio et al. 2002).

Recent attempts have successfully addressed the necessity for coordinated activity of several regulators within the neural crest specification network. Combining overexpression analyses in chick with examination of specification in the mouse embryo, Cheung et al. (2005) examined the role of neural crest specification genes belonging to three different families of transcription factors (Sox9, Snail2, and FoxD3) and addressed the hierarchical relationships among them. Their results suggest that proper specification of trunk neural crest cells requires concomitant activity of Sox9, Snail2 and FoxD3, with each transcription factor responsible for acquisition of a distinct feature: Sox9 establishes the competence to respond to induction signals that promote an EMT; Snail2 is responsible for the onset of the EMT program; and FoxD3 regulates expression of cell adhesion molecules required for neural crest migration (Cheung et al. 2005). These data are consistent with the idea that induction of neural crest segregation and initiation of an EMT are two separable events that occur in a coordinated manner, as has been previously suggested (Newgreen and Minichiello 1995; Sela-Donenfeld and Kalcheim 1999).

Neural crest specifiers control several downstream mediators of neural crest migration and differentiation (e.g. melanogenic and gliogenic neural crest cells; Figure
1.2C). Two (of many) interacting targets of neural crest specifier regulation, Rho GTPases and cadherins, mediate neural crest cell delamination by altering cell shape and adhesion (Fukata and Kaibuchi 2001). In chick, RhoB function is necessary for neural crest cell delamination and is upregulated in response to ectopic Snail2 (Liu and Jessell 1998; del Barrio and Nieto 2002). Cadherin-7 is upregulated as neural crest cells begin migrating and can be induced by ectopic FoxD3 expression (Nakagawa and Takeichi 1998; Dottori et al. 2001), while conversely, Cadherin-6B (a recently identified Snail2 direct target) is quickly downregulated at the onset of neural crest cell migration (Taneyhill et al. 2007).

**EMT and Cancer**

Numerous observations support the idea that EMT occupies a central role in tumor progression and cancer cell invasion. During progression to metastatic competence, carcinoma cells acquire expression patterns and express mesenchymal markers (e.g. N-cadherin, fibronectin). This course of action results in changed adhesive properties, and the activation of proteolysis and motility, which allows tumor cells to metastasize and colonize secondary tumors at distant sites (Sleeman 2000). In cell/tissue cultures, this progression is coupled with a partial or complete EMT, and induction of EMT in many carcinoma cell lines leads to the acquisition of metastatic properties in vivo (Huber et al. 2004; Moody et al. 2005). Furthermore, many factors that induce EMT in development are aberrantly expressed by cancer epithelial cells during tumorigenesis, incuding EGF, TGF-β, Rho GTPases, Snail1, Snail2, and Twist, just to name a few (reviewed in Huber et al. 2005). Metastasis is a complex, multi-step process, involving basement membrane destruction and local invasion, intravasation and
Figure 1.3: Sites of epithelial-mesenchymal transition during the tumor progression. (A) Normal epithelia (blue) lined by a basement membrane (red) can proliferate locally to give rise to an adenoma. Further transformation by epigenetic changes and genetic alterations leads to a carcinoma *in situ*, still outlined by an intact basement membrane. Further alterations can induce local dissemination of carcinoma cells (i.e. metastatic), likely through EMT, and the basement membrane becomes fragmented. (B) The invasive cancer cells (red) can intravasate (possibly also via EMT) into lymph or blood vessels, allowing them passively transport to distant organs. At secondary sites, individual carcinoma cells can extravasate and remain either solitary (micrometastasis) or form a new carcinoma (macrometastasis) through a mesenchymal-epithelial transition (MET). Figure adapted from Thiery, 2002.
Figure 1.3: Sites of epithelial-mesenchymal transition during the tumor progression
survival in the bloodstream, extravasation into distant organs and survival (plus proliferation) at the metastatic site (Pantel and Brakenhoff 2004) (Figure 1.3). Since the loss of E-cadherin expression or function is a hallmark of metastasis, most of the EMT-inducing developmental regulators (e.g. Snail and Twist) or pathways likely regulate one or more of these steps. It is not fully understood, however, at which steps epithelial plasticity changes are required. The transcription factor Twist was shown to be necessary for metastasis (Yang et al. 2004), apparently acting during emigration from the primary tumor and intravasation, while having no significant effect on the survival, extravasation or growth rate of tumor cells (Yang et al. 2004). Thus, EMT may be specifically involved in the movement of cells from a primary tumor into the circulation, suggesting that EMT is a necessary but not a sufficient step for forming overt metastases. It has recently been argued that EMT does not occur during tumor progression, and that the acquisition of mesenchymal properties simply reflects genomic instability (Tarin et al. 2005). However, it is very doubtful that the highly coordinated and complex gene-expression patterns that are required to endow tumor cells with the metastatic properties could arise through random mutations as a result of genomic instability. Rather, it would be more likely that genomic instability alters the expression of important developmental genes that regulate EMT.

**Sea Urchin EMT and Endomesoderm Network**

The four micromeres at the vegetal hemisphere of the sea urchin embryo appear at the 16-cell stage as result of an unequal fourth cleavage. At the onset of gastrulation, the micromere progeny (32 cells) undergo a phenotypic shift from epithelial cells to migratory mesenchyme cells (a classic example of EMT) to ingress into the blastocoel. These cells become primary mesenchyme cells (PMCs), and exclusively form the larval
skeleton later in embryogenesis. The easily identified lineage of the PMCs (established at 16-cell stage) and the predictability of their morphogenetic movement make the PMCs an excellent model for studying the mechanisms of EMT. Many previous studies have focused on the adhesion, matrix, and cytoskeletal changes occurring during the 30 minute period during which PMCs leave the epithelium and become mesenchymal (Fink and McClay 1985; Amemiya 1989; Burdsal et al. 1991; Anstrom 1992; Miller and McClay 1997; Ingersoll and Wilt 1998; Kanoh et al. 2001). One of the key characteristics of these PMCs is the loss of cell-cell adhesion during the EMT (Fink and McClay 1985), which is associated with the observed endocytosis of cadherin proteins (Miller and McClay, 1997).

The specification of the entire sea urchin endomesoderm, from which all mesoderm and endoderm arise, begins with the nuclear localization of β-catenin in the micromeres at fourth cleavage (Logan et al. 1999). Interference with nuclear localization and/or function of β-catenin by overexpressing cadherins, GSK3β or a dominant negative form of TCF/LEF can lead to a “dauer-blastula” phenotype, which almost lacks mesenchymal cells and gut completely (Emily-Fenouil et al. 1998; Wikramanayake et al. 1998; Logan et al. 1999; Veronica et al. 2000). The nuclear entry of β-catenin immediately activates a zygotic paired-box transcription factor, Pmar1, which specifies the micromeres (Oliveri et al. 2003; Nishimura et al. 2004) and functions to repress the expression of a ubiquitous repressor. This downstream ubiquitous repressor has been identified as HesC (Eric Davidson, personal communication). When ectopically expressed, Pmar1 is sufficient to convert all blastomerers into PMCs, demonstrating that Pmar1 is an upstream initiator of the entire micromere-PMC specification program (Oliveri et al. 2002). Several zygotic genes are under the control of Pmar1, including PMC transcription factors, Alx1 (Ettensohn et al. 2003), Ets1 (Kurokawa et al. 1999),
and Tbr (Croce et al. 2001; Fuchikami et al. 2002), as well as the Notch ligand Delta (Sweet et al. 2002) required for specifying secondary mesenchyme (discussed below). Once specified, the 32 PMCs will begin to ingress into the blastocoel (at approximately 10-11 hours post fertilization in *L. variegatus*)—the first event of gastrulation movements in sea urchin embryos.

Another cell population that also undergoes EMT in the sea urchin embryo is called secondary mesenchyme cell (SMC). SMCs are derived from macromere descendents, the large progeny from the unequal fourth cleavage that produced the micromeres. Several types of cells and tissues are derived from the SMCs, including pigment cells, blastocoelar cells, coelomic pouch cells, and circumesophageal muscles (Cameron et al. 1991; Ruffins and Ettensohn 1996). Specified beginning roughly at the eighth to tenth cleavage stages (Hörstadius, 1973; Cameron et al. 1991; Ruffins and Ettensohn 1993; Ruffins and Ettensohn 1996; Sherwood and McClay 1999; McClay et al. 2000), SMCs are the first cells to invaginate into the blastocoel as the primitive gut or archenteron is formed. SMCs stay at the tip of the archenteron until the mid-to-late gastrula stage, when they delaminate into the blastocoel via a similar ingression/EMT process seen with PMCs. Two sub-populations of SMCs, pigment cells and blastocoelar cells, have been shown to exhibit migratory behaviors (Gustafson and Wolpert 1967; Gibson and Burke 1985; Gibson and Burke 1987; Tamboline and Burke 1992). Newly delaminated pigment cells later invade the surrounding ectoderm (Gustafson and Wolpert, 1967; Gibson and Burke, 1985) and produce the echinochrome-containing pigment granules (McLendon, 1912; Griffiths 1965), while blastocoelar cells wander through the body cavity, forming complex connections through extended processes with the gut, skeletal rods, and ectoderm (Tamboline and Burke, 1992).

The Notch signaling pathway has been shown to play essential roles in SMC specification (Sherwood and McClay, 1999). One of the identified downstream targets
is Gcm, which controls the cell fates of pigment cells (Calestani et al. 2003; Ransick and Davidson 2006). However, transcription factors involved in regulating other SMC cell types, as well as genes involved in the EMT of SMCs, are currently not well understood.
Overview

This dissertation seeks to understand the mechanisms of PMC ingression in the sea urchin embryo and to construct an EMT regulatory network in the context of the existing micromere-PMC gene regulatory network (GRN). First, we identify a sea urchin orthologue of the Snail transcription factor, and focus on its roles in regulating EMT during PMC ingression. Our findings indicate that Snail plays an essential role in the EMT process by mediating the downregulation of cadherin expression, which is an evolutionarily conserved role for Snail. Additionally, our experiments uncover a new aspect of Snail’s function in regulating cell adhesion, where Snail positively controls the endocytosis of cadherin protein in PMCs. Second, we uncover the involvement of another mesoderm gene, Twist, in several developmental processes, including skeletogenesis and muscle differentiation. Although a requirement for Twist in mesoderm development is not novel, the finding that the sea urchin Twist is crucial for PMC ingression and fusion provides new perspectives to our understanding of how the PMC specification state is regulated. Lastly, utilizing the information from the recently annotated sea urchin (S. purpuratus) genome, several newly identified PMC genes were cloned and their expression patterns examined. Future experiments will focus on elucidating the roles of these genes in PMC ingression, as well as incorporating more genes into the PMC GRN with genomic approach. More importantly, our functional analysis of dynamically interconnected PMC subnetworks begins to reveal that an EMT process (or a specification event) is essentially coordinated by continuous but distinct regulatory network states. Overall, this work establishes the groundwork for unraveling the molecular mechanisms that regulate EMT during PMC ingression, as well as improving our understanding of how gene regulatory network states control a crucial morphogenetic event.
Chapter 2:

The Snail Repressor is Required for PMC Ingression in the Sea Urchin Embryos

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Summary

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 ingestion. Functional knockdown analyses of Snail in whole embryos and chimeras demonstrate that Snail is required in micromeres for PMC ingestion. 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 ingestion. Perturbation studies position Snail in the sea urchin micromere-PMC gene regulatory network (GRN), downstream of Pmar1 and Alx1, and upstream of several PMCexpressed 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 ingestion, and in part through its role in the endocytosis that helps convert an epithelial cell to a mesenchyme cell.
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 (Hörstadius, 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, \textit{alx1} (Ettensohn et al., 2003) and \textit{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 \textit{Drosophila} (Alberga et al., 1991), the first indication that the Snail family is involved in EMT came from \textit{Snail2} (\textit{Slug}) loss-of-function studies in chick embryos. Incubation of early chick embryos with antisense oligonucleotides to inhibit \textit{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 \textit{Snail} (\textit{Snai1} – Mouse Genome Informatics) is able to induce EMT when expressed in mammalian epithelial cells (Batlle et al., 2000; Cano et al., 2000), and \textit{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 TGF\beta, FGFs, EGFs, WNTs, BMPs and Notch, have been shown to 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’-AAAGACCCCTCGGCATCTTCTTGGATAA-3’; Oligo2: 5’-TTTTGACGAGAAAAAGACCTCGGCAT-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 \textit{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 \textit{Lvsnail} was amplified and cloned from a late-gastula stage cDNA pool (Accession Number DQ665364). \textit{Lvsnail} encodes a 337 amino acid protein based on the primary sequence data. ClustalW alignment between LvSnail and SpSnail proteins (\textit{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 \textit{snail} gene family possess 4-6 C\_H\_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 \textit{snail} family members from other species (Figure 2.1A) [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 \textit{Drosophila}, \textit{Caenorhabditis elegans} and \textit{Ciona} (Figure 2.1B), 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 \textit{snail} and \textit{slug} genes from vertebrates, amphioxus \textit{snail} (Bf \textit{snail}) and limpet \textit{snail} (Pv \textit{snail2}) (Figure 2.1C). The topology of this phylogenetic tree, as well as the distinct protein motifs, clearly supports the conclusion that \textit{Lvsnail} is an ortholog of \textit{snail} genes.
Figure 2.1: 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.
Figure 2.1: Sequence comparisons of Lytechinus Snail and related Snail family proteins
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 (Figure 2.2). No staining appears in early cleavage stages (Figure 2.2A), and expression is first detectable around the late-hatched blastula (HB) in the thickened vegetal plate region (Figure 2.2B). At early mesenchyme blastula (MB) stage, Lvsnail mRNA is expressed in ingressing PMCs (Figure 2.2C). The PMC expression of Lvsnail is reduced following ingression, and staining then appears within the SMC territory during archenteron invagination, and prior to ingression of SMCs (Figure 2.2D), then disappears from the ingressed SMCs. Throughout gastrulation, the SMC expression of Lvsnail persists at the tip of archenteron (Figure 2.2E), and exhibits both oral-aboral and left-right asymmetry at the midlate gastrula stage (data not shown). At prism stage, Lvsnail mRNA reappears in PMCs and becomes localized to the two ventrolateral PMC clusters (Figure 2.2F,G). The expression continues at these two sites, corresponding to the tips of the arm rods of the pluteus larva (Figure 2.2H). The SMC expression pattern of Lvsnail is consistent with the observations from a recent publication (Hardin and Illingworth, 2006).

LvSnail is required for PMC ingestion

To determine the function of LvSnail in sea urchin development, we designed and injected morpholino antisense oligonucleotides (SnaMASO) into fertilized eggs to block
Figure 2.2 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).
Figure 2.2 WMISH showing the dynamic pattern of Lvsnail mRNA expression during sea urchin development
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 Figure 2.3); 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 (Figure 2.4A, arrow), PMCs failed to ingress in Sna morphants (>90%, Figure 2.4D). The PMC ingression block continued (Figure 2.4F,G; 35%, 65%, respectively) even as control siblings completed gastrulation (Figure 2.4C). Although in a significantly delayed fashion, almost all Sna morphants eventually displayed a normal archenteron with no apparent phenotypic defects (see Figure 2.5). Other later phenotypes were observed in these Sna morphants, including loss of pigment cells, and stunted arm rod growth (see Figure 2.5), but in this study we focus only on the PMC ingestion phenotype.

At ingestion 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 dextran, shown in red) (Figure 2.6G). The resulting embryos showed no PMC ingression (Figure 2.6C,C’; 3/4, i.e. 3 out of 4 chimera embryos exhibited the phenotype shown in the figure), similar to Sna morphants (Figure 2.6B), whereas the reciprocal chimeric embryos (SnaMASO in animal half) developed normally (Figure 2.6D,D’; 4/4) compared with glycerol-injected controls (Figure 2.6A). As Lvsnail mRNA is expressed in ingressing PMCs, which are derived from the micromere lineage,
Figure 2.3: Sna morphant phenotypes are rescued by injection of MASO-resistant mRNA. (A) Control embryos. (B) Sna morphants show defects in PMC ingression. (C) Embryos at the same age as in B, co-injected with SnaMASO and ENG-Sna fusion mRNA (used as MASO-resistant mRNA) exhibit normal gastrulation with rescue of ingression (n=30, 40% rescue percentage). This experiment demonstrates the specificity of the SnaMASO used in this study.
Figure 2.3: Sna morphant phenotypes are rescued by injection of MASO-resistant mRNA
Figure 2.4: PMC ingestion is blocked by SnaMASO injection. (A-C) Control embryos show normal PMC ingestion (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 ingestion (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.
Figure 2.4: PMC ingress is blocked by SnaMASO injection
Figure 2.5: Pigment cells are reduced in Sna morphants. (A, B) Control embryos showed normal numbers of pigment cells (Avg. 82.3±6.8). (C, D) Compared to the control, Sna morphants showed significantly reduced pigment cells (Avg. 40.2±5.0), and also showed stunted growth of anal arm rods.
Figure 2.5: Pigment cells are reduced in Sna morphants
Figure 2.6: 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.
Figure 2.6: Chimeric embryos demonstrate Snail is required in micromeres for ingresson
we then replaced a single micromere from a green-dyed control host with a SnaMASO-injected micromere (in red) (Figure 2.6H). The red micromere progeny failed to ingress (Figure 2.6E’, arrow), whereas the green micromere progeny (serving as internal controls) ingressed normally and settled at the bottom of the blastocoele (Figure 2.6E,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 (Figure 2.6F,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 (Figure 2.7). 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 (Figure 2.7E,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 (Figure 2.7G). 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 (Figure 2.7A-D) and corroborated by QPCR (data not shown), the expression level of both genes did not
Figure 2.7: 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 ΔC\textsubscript{T} ± s.e.m.
Figure 2.7: Effects of SnaMASO on PMC specification and differentiation
change in Sna morphants. Moreover, they continued to be expressed in the PMC precursors in the central region of the vegetal plate (Figure 2.7B,D), even though in the absence of Snail, the PMCs failed to ingress (Figure 2.7A,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 ingress, and exhibited reduced mRNA expression of Lvsnail as shown by WMISH (Figure 2.8A,B). In U0126-treated embryos, however, the expression of Lvsnail appeared to be normal regardless of the block to PMC ingress (Figure 2.8C,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.

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

<table>
<thead>
<tr>
<th>Perturbation</th>
<th>Snail (ΔCt)</th>
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<tbody>
<tr>
<td>U0126-treated</td>
<td>NS/NS, NS</td>
</tr>
<tr>
<td>DN-Ets1 MOE</td>
<td>NS</td>
</tr>
<tr>
<td>AlxMASO</td>
<td>-2.54/-3.4, NS</td>
</tr>
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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.
Figure 2.8: 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).
Figure 2.8: Upstream regulators of LvSnail
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 (Figure 2.9B), and overexpression of LvSnail alone results in an excess number of mesenchyme cells in the blastocoele (Figure 2.9, 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, Figure 2.9D), 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 a FITC-injected control embryo (to serve as an experimental control) or from an AlxMASO/Snail co-injected embryo (dyed green) (Figure 2.9E). 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 (Figure 2.9F-G). In controls, all green micromeres ingressed into the blastocoele (6/6) (Figure 2.9F-F’). In the double-injected experimental set, more than half of the AlxMASO/Snail co-injected green micromeres ingressed (15/27) (Figure 2.9G-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.
Figure 2.9: 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 blastocele, whereas the AlxMASO-injected micromere (red) fails to ingress. Brightfield (G), fluorescent (G’), and merged (G”) images are shown.
Figure 2.9: LvSnail functions downstream of LvAlx1 in PMCs
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 (Figure 2.10A), most cells of the *Lvpmar1*-injected embryo were converted into PMCs when *Pmar1* was ectopically expressed (Figure 2.10B), 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 (Figure 2.10D). WMISH of *Lvsnail* showed a ubiquitous expression, also as might 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, Figure 2.10C), 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 (Figure 2.10B). Thus, ectopic expression of *Pmar1* requires Snail expression to complete the transformation and ingression of ectopic cells in the embryo.
Figure 2.10: 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 Gcadherin 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.
Figure 2.10: LvSnail downregulates G-cadherin expression and positively regulates its endocytosis
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 (Figure 2.9C; 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 (Figure 2.10C). This experiment demonstrates, therefore, that Snail downregulates the expression of Cadherin at PMC ingressoin in sea urchin embryos.

LvSnail positively regulates cadherin endocytosis in PMCs

At PMC ingressoin, 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 (Figure 2.10E) and also forms punctate foci of the intracellular GFP signal when endocytosed in ingressing PMCs (arrowheads in Figure 2.10F,G). Using this construct the following experiment was performed as shown in Figure 2.10H. 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 Figure 2.10H). In about 60% of SnaMASO two-cell injection embryos ($n=70$), SnaMASO successfully blocked PMC ingression 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 (Figure 2.10F,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 SnaM ASO blocks the first event in PMC morphogenesis (EMT/ingression) (Figure 2.4), as well as the expression of several PMC differentiation genes (Figure 2.7G). Second, the SnaMASO chimeric experiments demonstrate that Snail function is necessary in micromeres for ingression to occur (Figure 2.6), as Snail-deficient micromere descendants do not migrate into the blastocoele of a normal host embryo, and stay at the tip of the archenteron (Figure 2.6E,E’); absence of Snail everywhere else in the embryo has no effect on the Snail-expressing micromere, which retains its ability to ingress (Figure 2.6F,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 Barral-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 ingestion), 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 (Barral-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 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 (Figure 2.10), 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 ingestion (Miller and McClay, 1997a), and this appears to be positively regulated by Snail (as shown in Figure 2.10E-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.
*Lonlail* 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 Figure 2.11). 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 Figure 2.12).

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 (Figure 2.10D), 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 (Figure 2.8A,B), whereas Alx1 expression is unaffected in Sna morphants (Figure 2.7A,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-depleted micromeres in about 60% of the chimera embryos (Figure 2.9). This partial rescue suggests that Alx1 regulates PMC ingression partly through Snail but may require other unknown gene
Figure 2.11: Chimera experiments show that ingression of SMCs also requires Snail.

(A-C) Experimental control (control micromeres combined with control host, n=13).
Arrows show the SMC cells (pigment cells or blastocoelar cells). Control PMCs shown in
green (B'). (C) Control chimera containing a normal number of pigment cells. (D-F)
Chimera embryos (n=13) with control micromeres (in green, shown in E') in a SnaMASO-
host (in red, as an injection tag, not shown), which the SnaMASO is present in
macromeres. The migrating SMCs are rarely observed at the tip of the archenteron of the
chimera embryo (D), when compare to control embryo (A). Fewer SMCs are seen in the
blastocoel even at later stages (E, F). (G) Quantification of SMC numbers shows that
Snail is involved in SMC ingression. At LG stage, any free mesenchyme cells that were
not PMCs (not dyed green) were collectively counted, as these either could be
blastocoelar cells or pigment cells (labeled as BC+PC). 1st and 2nd indicates two
separate experiments (total n=13). At the 48hr pluteus stage, the pigment cells are
distinguishable as they appear in red, while blastocoelar cells could be counted also as
cells free in the blastocoel and not green PMCs. The cell numbers of SMCs are
significantly lower in these chimera embryos than in the controls.
Figure 2.11: Chimera experiments show that ingression of SMCs also requires Snail
Figure 2.12: 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.
Figure 2.12: Model of a Snail-dependent pathway regulating PMC ingression in the PMC-micromere GRN
targets, thereby preventing a full rescue phenotype. Nevertheless, the rescue observed here further validates the designated position of Snail in the PMC GRN (Figure 2.12). 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 (Figure 2.7C,D), and Snail mRNA expression is also unaltered in U0126-treated embryos (Figure 2.8C,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 Figure 2.12).

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 (Figure
2.2F,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 (Figure 2.12). 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 ingress.

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 ingress in the sea urchin, and also provide useful insight into the complex molecular strategies that regulate EMT events in other organisms.
Chapter 3:

Multiple Roles of the Twist Transcription Factor in Mesoderm Formation during Sea Urchin Embryogenesis
Introduction

The specific cellular movements of gastrulation, establish a unique cell layer – the mesoderm – between the endoderm and the ectoderm. In sea urchin embryos, the mesoderm consists of two different populations of cells, primary and secondary mesenchyme cells (PMCs and SMCs, respectively). PMCs exclusively form larval skeletons, while SMCs divide into four sub-populations, i.e. pigment cells, esophageal muscles, blastocoelar cells, and coleomic pouch cells.

Micromeres, the PMC precursors, appear at 16-cell stage as a result of an unequal cleavage in the vegetal hemisphere. During early cleavage, they become autonomously specified (Hörstadius, 1973; Okazaki, 1975; Davidson 1989; McClay et al. 1992; Ransick and Davidson 1993; Davidson et al. 1998) and later penetrate through the basal lamina via ingestion, a classic epithelial-mesenchymal transition (EMT) process, into the blastocoel (Katow and Solursh 1980). In the micromere-PMC gene regulatory network (GRN), several transcription factors have been shown to be essential for the early specification [pmar1 (Oliveri et al. 2003), alx1 (Ettensohn et al. 2003), ets1 (Kurokawa et al. 1999)], PMC differentiation [tbr (Fuchikami et al. 2002), dri (Amore et al. 2003), hnf6 (Otim et al. 2004)], and recently, the EMT/PMC ingestion [snail (Wu and McClay 2007)]. SMCs, on the other hand, initiate specification as a result of the Notch signaling pathway (Sherwood and McClay 1999; Sweet et al. 2002), which activates SMC specification genes beginning at about 7\textsuperscript{th} cleavage. The earliest known gene in this specification is gcem (Ransick and Davidson 2006). Later, some of the SMCs will undergo EMTs that appear similar to the PMC ingestions, but the SMCs separate from the tip of the archenteron at the gastrula stage. The underlying molecular mechanisms or the genes involved in this EMT, and the relationship between the two EMTs is largely unrevealed so far. To further understand the mesoderm (PMC and SMC)
specification and morphogenesis in sea urchins, we decided to investigate the function of a candidate gene, twist, which is well documented for its evolutionarily conserved roles for mesoderm development (see review in Castanon and Baylies 2002).

The twist gene was first identified in Drosophila as the twist mutant embryo exhibited a twisted torso (Simpson 1983; Nusslein-Volhard et al., 1984). Extensive studies in Drosophila have shown that Twist activity is crucial for many aspects of embryogenesis, such as the establishment of dorsoventral tissue patterning, the specification of mesodermal fate, and myogenesis (Thisse et al. 1987; Leptin 1991; Baylies and Bate 1996; Cripps and Olson 1998). Other studies in vertebrates and C. elegans continue to support the notion that Twist is generally involved in the patterning of mesodermal tissue fates, especially for compartmentalization of muscle development. For example, the C. elegans Twist homolog, hlh-8, plays a critical role in the formation of non-striated muscles (Corsi et al. 2000) while Twist<sup>-/-</sup> mutant mouse embryos display severe defects in closure of the cephalic neural tube, deficient mesoderm, malformed branchial arches, defective cranial neural crest cell migration, and retarded development of limb bud (Chen and Behringer 1995; O’Rourke et al. 2002; Soo et al. 2002; Zuniga et al. 2002). Twist-null heterozygous mice (Twist<sup>+</sup>/) exhibit similar phenotypes of the dominantly inherited Saethre-Chotzen syndrome in the human population (Bourgeois et al. 1998), which is possibly due to the haplo-insufficiency of Twist allele (el Ghouzzi et al. 1997; Howard et al. 1997). In addition to its roles associated with mesoderm specification and differentiation, Twist has been implicated as an EMT regulator (Rosivatz et al. 2002) for its role in activating N-cadherin during Drosophila embryogenesis (Oda et al., 1998). Moreover, a recent study investigating Twist’s role in tumor progression notably sustains and enhances this notion (Yang et al. 2004). Forced Twist expression is sufficient to induce phenotypic and molecular hallmarks of an EMT in different cell lines (Yang et al. 2004). Furthermore, the nuclear translocation of Twist
protein also impacts cell migration during tumor metastasis (Alexander et al. 2006). In sea urchin embryos, the PMCs and SMCs are excellent models for studying EMT and cell fate specification in vivo, and with the expanding sea urchin endomesoderm GRN (Davidson et al. 2002), we can investigate if sea urchin Twist plays a similar role during mesoderm formation. More importantly, we can examine Twist’s functions in the context of dynamic gene network states to further comprehend its association with morphogenetic processes, such as PMC ingestion.

In this study, we report the identification, characterization, and functional analyses of *Lvtwist*; a member of the Twist family of transcription factors in *Lytechinus variegatus*. We show that *Lvtwist* is required for the micromeres to undergo the proper EMT process to ingress into the blastocoel. Moreover, *Lvtwist* positively regulates PMC differentiation and skeletogenesis. We further attempt to fit *Lvtwist* into the current version of the micromere GRN, and examine its roles in SMCs development as well.
Materials and Methods

Animals and drug treatments

*Lytechinus variegates* adults were obtained from 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 *Lvtwist*

The coding sequence of *Lvtwist* was obtained by RT-PCR from a *Lytechinus variegatus* gastrula cDNA library (GenBank Accession Number XXXXX). All PCR products were cloned into a PCS2 vector for mRNA synthesis.

In situ hybridization

In situ hybridization was performed using standard methods with DIG-labeled RNA probes and BM purple substrate (Roche) for detection (see methods in Wu and McClay, 2007). Hybridizations and washes were carried out at 60-65°C depending on the probes. Two *Lvtwist* probes were used; one corresponds to the *Lvtwist* full-length ORF and the other one was the ORF plus a stretch of 3’UTR sequence. Other probes were synthesized from the *LvetS1, Lvalx1* and *Lvsnail* clones (Wu and McClay, 2007).

Morpholino antisense oligonucleotides (MASO) and mRNA injections

Two non-overlapping *Lvtwist*-specific MASOs were obtained from Gene Tools, and both have the same efficiency at 1 to 1.5 mM. (Oligo1: 5’-TTCTTCTCGGCG-CGTGAACCATT-3’; Oligo2: 5’-CGTACTCGTACCCTCCGCAGTAAAC-3’). 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. Double injections were performed by injections of Pmar1 mRNA and TwiMASO simultaneously at the concentrations indicated above.

Transplantation experiments

Micromere transplantations were performed at 16-cell stage, with *L. variegatus* embryos. Detailed procedures were followed as previously described (Logan et al., 1999). For the PMC fusion experiments, two micromeres were transplanted inside the blastocoel of the 32- or 60-cell stage, and most procedures are similar as micromere swapping experiments (Wu and McClay, 2007).

Immunostaining

Embryos were methanol-fixed, stained with 1d5 mAb (1:200) or anti-β-catenin pAb (1:50) or anti-myosin pAb (1:750) in 4% normal goat serum/PBS, and incubated overnight at 4°C. After washing three times in PBS, samples were incubated with Cy2, Cy3 or Cy5-conjugated secondary antibodies (Jackson Immunoresearch Laboratories) for 1-2 hour at room temperature, and then imaged as 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 transcriptase (RT) reactions 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 \textit{Lvtwist}

The \textit{twist} gene belongs to a diverse group of transcription factors that share a common basic helix-loop-helix (bHLH) motif. The bHLH domain was first recognized in the murine DNA-binding proteins E12 and E47 (Murre et al. 1989), and subsequently identified in a number of proteins encoded by many genes that play important roles in cell specification, tissue differentiation, and growth regulation (see review in Jan and Jan 1993).

Using RT-PCR and RACE, the open reading frame of \textit{Lytechinus variegatus twist} (\textit{Lvtwist}) was amplified and cloned from a gastula stage cDNA pool. \textit{Lvtwist} encodes a 201 amino-acid polypeptide based on the primary sequence data. Although \textit{twist} was not annotated in the recent published genome of \textit{Strongylocentrotus purpuratus} (Howard-Ashby et al. 2006; Sodergren et al. 2006), we successfully identified the putative \textit{Sptwist} gene, which encodes a 204 amino acid polypeptide, by blasting the assembly of \textit{S. purpuratus} genome with the coding sequence of \textit{Lvtwist}. The ClustalW pairwise alignment between \textit{LvTwist} and \textit{SpTwist} shows that two proteins share an overall amino acid identity of 92\% (data not shown), despite being separated by 30-40 million years.

Members of \textit{twist} gene family have been identified in different species throughout evolution, including jellyfish (PcTwist; Spring et al. 2000), sea anemone (NvTwist; Martindale et al. 2004), and \textit{C. elegans} (CeTwist, \textit{hlh-8}; selectively excluded from our analysis due to its substantial divergence; (Harfe et al. 1998). Multiple sequence alignment of Twist proteins shows that \textit{LvTwist} and other Twist family members are highly conserved within the bHLH domain (Figure 3.1A). Furthermore, \textit{LvTwist} is
Figure 3.1: Sequence comparisons of *Lytechinus* Twist and related Twist family proteins. **(A)** The bHLH DNA-binding domain of *L. variegatus* Twist (*LvTwist*) compared to related proteins in other organisms. **(B)** The WR domain of *LvTwist* matches the consensus and is identical to those of most *twist* family members, including *Nematostella*, mouse and *Xenopus* Twist. **(C)** Rooted neighbor-joining tree showing the relationship of *LvTwist* with other *twist* family proteins (1000 bootstraps, values indicated on nodes). *Nematostella* and *Podocoryne* Twist served as outgroups.
Figure 3.1: Sequence comparisons of Lytechinus Twist and related Twist family proteins
identical to almost all Twist proteins except *Drosophila* Twist (DmTwist) and amphioxus Twist (BbTwist; Yasui et al. 1998) at all 14 residues of the WR motif (Figure 3.1B; ERLSYAFSVWRMEG), which is a C-terminal motif characteristic for the Twist protein family (Spring et al. 2000). Phylogenetic analysis based on Twist protein sequences (Figure 3.1C), as well as the strong conservation of distinct protein motifs, strongly supports the orthology of *Lvtwist* to other twist family genes.

*Lvtwist* mRNA is expressed in mesoderm during gastrulation

A temporal expression profile of *Lvtwist* mRNA (Figure 3.2A) showed the expression level of *Lvtwist* remained relatively low during early stages until about the hatched blastula stage with a transient increase, and started to accumulate after MB stage.

Whole-mount in situ hybridization (WMISH) showed that *Lvtwist* mRNA is expressed mainly in the mesoderm, PMCs and SMCs (Figure 3.2B-E). At early mesenchyme blastula (MB) stage, *Lvtwist* mRNA is expressed in ingressing PMCs (Figure 3.2B). The PMC expression of *Lvtwist* is maintained after MB stage and throughout gastrulation (Figure 3.2C-E). *Lvtwist* expression is also observed in the SMC territory (and maybe endoderm too) at EG stage (Figure 3.2D), and at the tip of archenteron at LG stage (Figure 3.2E).

LvTwist is required for proper PMC ingression, then for skeletogenesis

To determine the function of LvTwist in sea urchin development, we designed and injected morpholino antisense oligonucleotides (TwiMASO) into fertilized eggs to interfere with endogenous LvTwist translation. To test the efficiency of the morpholino used in this study, co-injection of either the TwiMASO or a standard control morpholino (from Gene Tools) and a GFP construct containing the target sequence complementary to
Figure 3.2: The temporal and spatial expression patterns of *Lotwist* mRNA during sea urchin embryogenesis. (A) A temporal expression profile by QPCR shows the dynamic expression levels of *Lotwist* mRNA. The two dotted blue lines indicate 150 and 350 transcripts (below and above, respectively). (B-E) WMISH shows the expression domains of *Lotwist* mRNA. *Lotwist* mRNA is detected in the early ingressing PMCs (arrow in B) and ingressed PMCs (C). (D) Early gastrula stage (EG): *Lotwist* mRNA expression persists in PMCs (arrow), and is also expressed in SMCs. (E) Late gastrula stage (LG): *Lotwist* mRNA continues to be expressed in PMCs and the SMC territory.
Figure 3.2: The temporal and spatial expression patterns of *Lvtwist* mRNA during sea urchin embryogenesis
the morpholino was performed. The TwiMASO specifically blocked the GFP expression (data not shown), and injecting the control morpholino at the same or higher concentration than the TwiMASO had no effect on development; moreover, a second Twist-specific morpholino (see Materials and Methods) also exhibited the same phenotype as the morpholino reported here.

TwiMASO-injected embryos (‘‘Twi morphants’’) developed normally through the early cleavage stages, and they hatched normally as controls. However, when PMCs of control embryos ingressed into the blastocoel at MB stage (Figure 3.3A, arrow), PMCs failed to ingress in Twi morphants (>80%, Figure 3.3E) showing a significant delay. The delay of PMC ingestion continued (Figure 3.3F) until control injected siblings reached mid-gastrula stage (Figure 3.3B). When control embryos completed gastrulation (LG/PR stage, Figure 3.3C), PMCs in Twi morphants started to make their way into the blastocoel and the archenteron started to invaginate (Figure 3.3G). In later stages, PMCs eventually formed a ring around the archenteron but no spicules were observed in Twi morphants (Figure 3.3H,H’), while control embryos at pluteus stage showed normal skeletal patterns (Figure 3.3D). To confirm the skeletal phenotypes in Twi morphants, we cultured the embryos for longer periods to see if spiculogenesis occurs (Figure 3.4). At 72hrs, control embryos exhibited normal and elaborate skeletal patterns (Figure 3.4A-B’), while Twi morphants had no larval skeleton formed (Figure 3.4C-D’). Other phenotypes were also observed such as reduction of pigment cells (an effect on SMCs) (Figure 3.4C,D; data not shown), and failure of the archenteron elongation although considered secondary effects (~25%, Figure 3.4C). Taken together, these phenotypes of Twi morphants strongly suggest that twist is involved in PMC (including ingestion and later skeletogenesis) and SMC development.
Figure 3.3: PMC ingression and skeletogenesis are impaired by TwiMASO injection.

(A-D) Control embryos show normal PMC ingression (arrow) with normal larval skeletons (arrowheads). (E-H') Embryos injected with TwiMASO. Compared to the control, TwiMASO-injected embryos (Twi morphants) show delayed PMC ingression (E-G), and no skeleton (H), as revealed by polarized light (H'). Stages: MB, mesenchyme blastula; EG, early gastrula; MG, mid-gastrula; LG, late gastula; PR, prism; PL, pluteus larvae.
Figure 3.3: PMC ingression and skeletogenesis are impaired by TwiMASO injection
Figure 3.4: Skeletogenesis is persistently blocked in Twi morphants. (A-B’) Control embryo at 72 hours post-fertilization (hpf). The skeletal elements are clearly visible under polarized light (B’). (C-D’) Twi morphants at 72 hpf. The larval skeleton is absent in Twi morphants. Note that the archenteron elongation is also defective, although only observed in about 25% of the embryos. PL, pluteus larvae stage.
Figure 3.4: Skeletogenesis is persistently blocked in Twi morphants
LvTwist functions autonomously in micromeres for PMC ingression

To further examine the impact of LvTwist on PMC ingression, chimeric embryos were generated as a useful experimental method to localize the function of a gene (Wu and McClay, 2007; also shown in Figure 3.5C). We replaced a single micromere from a control host (with FITC, shown in green) with one TwiMASO-injected micromere (with rhodamine-conjugated dextran, shown in red) (Figure 3.5A,A’). The red micromere progeny seemed to initiate ingression but failed to ingress completely and somehow remained attached to the archenteron (Figure 3.5A’, arrows), while the green micromere progeny (serving as internal controls) ingressed normally and settled at the bottom of the blastocoel (Figure 3.5A,A’; arrowheads). The reciprocal experiment showed that progeny of a single green control micromere ingressed normally even when placed onto a red-dyed TwiMASO-injected host (Figure 3.5B,B’; arrow). In conclusion, these chimeras show that Twist is essential in micromeres for these cells to ingress completely and punctually into the blastocoel as PMCs.

LvTwist functions to maintain the specification program of the micromeres

To achieve a better molecular understanding of the effects of Twist on PMC formation, we examined the expression of key genes of the micromere GRN in Twi morphants (Figure 3.6). First, Twi morphants failed to stain with 1d5, a monoclonal antibody that recognizes a PMC-specific cell surface MSP130 glycoprotein, when control embryos at MB stage showed prominent staining (Figure 3.6G,H). The mRNA expression level of Lvmsp130, Lvsm30, and Lvsm50 were also examined by QPCR, and were all significantly reduced in Twi morphants, when compared to controls (Table 2). These data suggest that LvTwist functions upstream of PMC skeletogenic differentiation program, but the interactions could be either direct or indirect.
Figure 3.5: Chimeric embryos demonstrate Twist is required in micromeres for ingestion. (A-A’) Single TwiMASO-containing micromere (red) transplanted onto a control host embryo lacking one micromere (green). The TwiMASO micromere failed to ingress completely and spread along the archenteron (arrows in A’), while endogenous control micromeres ingressed and migrated normally (arrowheads). (B-B’) The reciprocal experiment to that in A. One normal micromere (green) ingressed into the blastocoel (arrow in B’) when transplanted to a TwiMASO-injected host embryo lacking one micromere (red). (C) The schematic diagram of the experimental designs of A and B. See text for details.
Figure 3.5: Chimeric embryos demonstrate Twist is required in micromeres for ingestion
Figure 3.6: Effects of TwiMASO in PMC specification and differentiation. (A-F) In situ hybridizations with *Lvalx1, Lvets1* and *Lvsnail* probes. Control mesenchyme blastula stage embryos show normal expression of these three genes in PMCs (A,C,E).

Expression of *ets1* is not affected in Twi morphants (D) but expression of *alx1* and *snail* is reduced (B,F). (G-J) Immunostaining with PMC mAb 1d5, shows the strong presence of 1d5 in controls (G,I), but no expression of 1d5 detectable in Twi morphants (H,J). Anti-β-catenin staining was used to outline the cell boundaries (I,J). MB, mesenchyme blastula stage; LG, late gastrula stage.
Figure 3.6: Effects of TwiMASO in PMC specification and differentiation
Three transcription factors are known to be essential in the micromere-PMC GRN; for specifying early micromeres, *alx1* (Ettensohn et al. 2003) and *ets1* (Kurokawa et al. 1999), and for regulating EMT of PMC ingression, *snail* (Wu and McClay 2007). We measured the mRNA expression level of these genes in the presence of TwiMASO by WHISH and QPCR to see if Twist regulates the expression of any these genes. In the absence of Twist, the PMCs failed to ingress (Figure 3.5B,D,F), and *alx1* showed a reduced level of expression in Twi morphants, when compared to controls (Figure 3.6A,B; Table 2). However, in Twi morphants, *ets1* continued to be expressed in the PMC precursors in the central region of the vegetal plate (Figure 3.6C,D; Table 2). On the other hand, the expression level of *snail* mRNA also diminishes significantly as shown by QPCR (Table 2) and WMISH (Figure 3.6E,F). These data indicate that *twist* plays an important role in the micromere-PMC GRN. It is independent of *ets1* specification and somehow connected to the *alx1 to snail* regulatory pathway (Wu and McClay 2007). Pmar1, a transcriptional repressor, is necessary for early initiation of the micromere specification program, and essentially upstream of the entire PMC GRN (Oliveri et al. 2011).
Ectopic expression of Pmar1 transforms most cells of the embryo into PMCs (Oliveri et al. 2002). Therefore, if Twist is indeed required for EMT, we expect that TwiMASO would block this Pmar1 ectopic-expression phenotype, as shown before for snail (Wu and McClay 2007). Compared to a normal control (Figure 3.7A), almost all cells of the Lvpmar1-injected embryo were converted into PMCs when Pmar1 was ectopically expressed (Figure 3.7B). When embryos were co-injected with Pmar1 and TwiMASO, the resulting embryos showed a wide range of phenotypes (n=100, Figure 3.7D-F). About 50-60% of the co-injected embryos still showed massive mesenchymal extrusion (Figure 3.7D), while the remaining embryos either resembled wild-type embryos (~10%; Figure 3.7A,E) or exhibited a delayed ingression as observed in Twi morphants (~30%; Figure 3.7C,F). Thus, ectopic expression of pmar1 only partially requires Twist expression to complete the transformation of ectopic PMCs in the embryo.

LvTwist is necessary for PMC fusion

In Twi morphants, skeletogenesis was severely attenuated (Figure 3.4), which could have resulted from disruptions of several different developmental processes, including PMC differentiation or PMC fusion. The staining of PMC-specific antibody, 1d5, was significantly reduced in Twi morphants when compared to controls at MB stage (Figure 3.6G,H) and LG stage (Figure 3.6I,J) control embryos. These results suggest that TwiMASO affects the skeletogenesis by somehow preventing completion of the PMC differentiation program. The next experiments attempted to determine at what level skeletogenesis was disrupted.

PMC filopodia undergo syncytial fusion to form a cable-like structure within which the spicules are subsequently secreted (Hodor and Ettensohn 1998). Because PMC fusion and the formation of the filopodial cables are required for normal
Figure 3.7: TwiMASO can not fully rescue the ectopic-Pmar1 phenotype. (A) Control late gastrula stage embryos. (B) Embryos with ectopic-expressed Pmar1 produce extra PMCs and display massive mesenchyme extrusions. (C) Twi morphants exhibit significantly delayed PMC ingression as compared to controls (A). (D-F) Pmar1/TwiMASO co-injected embryos show a wide spectrum of phenotypes, from no rescue (D) to fully rescued (F) embryos.
Figure 3.7: TwiMASO can not fully rescue the ectopic-Pmar1 phenotype
skeletogenesis, we hypothesized that Twist is required for PMC fusion. When extra PMCs are implanted into the blastocoel, they normally fuse with endogenous PMCs (Ettensohn 1990). To determine whether Twist is required for PMC fusion we transplanted two micromeres into the cavity of a 32- (or 60-) cell stage embryo to purposely include these micromeres as “ingressed” in the blastocoel and thereby bypass the PMC ingression process. The resulting chimeras with an “ectopic micromere” were shown in Figure 8. When two control micromeres (one in red, and one in green) were transplanted, progeny of both exhibited normal fusogenic behavior (Figure 3.8A-C) and differentiation (as shown in 1d5 staining; Figure 3.8D). However, when one TwiMASO-injected micromere (in red) and one control micromere (in green) were transplanted, as expected, the progeny of the normal micromere fused with endogenous PMCs, while the TwiMASO-micromere progeny failed to enter the syncytium with other PMCs (Figure 3.8E-G), and did not stain with 1d5, either (Figure 3.8H). Taken together, these data support the hypothesis that Twist is required for proper PMC fusion, and therefore for developmental events upstream of skeletogenesis.

**LvTwist is involved in SMC development**

The expression of Twist mRNA by WMISH is also observed in SMC territory, so it would be reasonable to hypothesize that Twist plays a role in SMC development. In Twi morphants, the number of pigment cells was significantly decreased when compared to the control embryos (Figure 3.4A-D; data not shown). Other populations of SMCs were also examined, especially muscle cells, in Twi morphants, since twist family genes had been implicated in many aspects of muscle development (Castanon and Baylies 2002). Control embryos at the 27hr-pluteus larvae stage have muscle cells/fibers that surround the esophagus of the sea urchin embryo, and can be visualized by staining with myosin antibody (Figure 3.9A). We found that there was no myosin antibody staining in
Figure 3.8: Chimera embryos indicate that Twist activity is critical in micromeres for proper PMC fusion. (A-D) PMCs derived from two transplanted control micromeres (red and green) are equally capable of fusing with endogenous PMCs (no color) and express the MSP130 glycoprotein (D, shown by 1d5 staining). (E-H) PMCs derived from a TwiMASO-injected micromere (red) fail to fuse properly with endogenous PMCs, while control micromere-derived PMCs (green) fuse normally. The 1d5 staining is absent in the TwiMASO-containing PMCs (H). The schematic diagram of the experimental designs of A to H is shown in I. See text for details.
Figure 3.8: Chimera embryos indicate that Twist activity is critical in micromeres for proper PMC fusion
Figure 3.9: Injection of TwiMASO affects muscle development in sea urchin embryos. (A) Control 27-hr pluteus larvae embryos show normal muscle formation (anti-myosin), normal PMC pattering (1d5) and differentiated archenteron (endo1). (B,C) The anti-myosin staining is absent in Twi morphants as well as the staining of the endoderm marker, endo1. 1d5 staining indicates that PMCs are differentiated, but highly disorganized.
Figure 3.9: Injection of TwiMASO affects muscle development in sea urchin embryos
Twi morphants (Figure 3.9B,C), which suggests that Twist is necessary in the SMC lineage in the specification series leading to muscle formation in sea urchins.
Discussion

We have shown that *twist* gene is involved in execution several different functions in mesoderm development during sea urchin embryogenesis (summarized in Figure 3.10). One of these functions is maintaining a stable state of PMC specification, which enables PMCs to undergo a complete EMT and form the larval skeleton. Moreover, *twist* also is necessary for PMC fusion and muscle development, both of which are under-explored areas in the sea urchin development. It is likely that each of the Twi morphant phenotypes occurs as a consequence of incomplete specification prior to ingression. With the well-established endomesoderm GRN, our study here uncovered the multiple downstream roles for *twist* in regulating both the specification and morphogenesis in different mesodermal cell lineages, but the exact position in the mesoderm gene regulatory network remains to be established.

Fitting Twist in the GRN and connecting the network to morphogenesis

Twist expression occurs prior to ingression, well after initial expression of Alx1, a transcription factor operating early in the micromere GRN. Because loss of Twist expression results in a later reduction of Alx1 activity, and has no effect on Ets1, Twist is probably involved in maintaining a branch of the network involving Alx1. Alx1 is known to be necessary for ingression and for further differentiation of PMCs (Ettensohn et al. 2003) and the Twist morphant phenotypes further support the connection to this branch of the PMC GRN. Somehow the early specification leads to morphogenesis, so the challenge is to connect branches of the micromere gene regulatory network to the many aspects of morphogenesis that follow that specification. This currently is a great challenge because it means connecting what is largely a transcription regulator network
Figure 3.10: The different functions of Twist during mesoderm development in sea urchin embryos. Proposed diagram showing Twist mesodermal activity: After PMC regulatory genes (light blue box; several genes omitted), Alx1 and Ets1 are activated by Pmar1, they in turn to regulate skeletogenic differentiation genes (dark blue box) and EMT genes (red box) for PMC ingestion, such as Snail. Twist is involved in several developmental processes, such as PMC ingestion, skeletogenesis, and PMC fusion, possibly via regulating the expression of Alx1, Msp130, and Snail (temporally; gray arrow). On the other hand, Pmar1 de-repression system also turns on Delta, which induces SMCs (green box) through Notch signaling, and activates Gcm for pigment cells. Unknown regulators (denoted as X) for muscle development are also under the control of Notch signaling. Twist may regulate muscle formation through one (or several) of these genes. HB, hatched blastula stage; MB, mesenchyme blastula stage.
Figure 3.10: The different functions of Twist during mesoderm development in sea urchin embryos
to many cellular functions collectively called morphogenesis. Below is a synopsis of those cellular functions and possible roles of Twist in regulation of them.

**Sea urchin Twist function is essential for proper PMC ingression**

Several independent observations support the conclusion that Twist function is involved upstream of PMC ingression but not essential for the occurrence of ingression. First, functional knockdown of Twist with TwiMASO significantly delays PMC ingression (Figure 3.3), though ingression eventually occurs. Second, Twist knockdown significantly delays or blocks overall expression of different PMC differentiation markers (Figure 3.6G-J; Table 2). Third, the micromere-swap chimeric experiments demonstrate that Twist function is necessary autonomously in micromeres for a complete ingression (Figure 3.5), since the TwiMASO-injected micromere progeny (from a single micromere) do not migrate all the way into the blastocoel, and scatter throughout the upper half of the archenteron (Figure 3.5A,A'); the TwiMASO-containing host has no effects on the ingression ability of the control micromeres (expressing Twist) (Figure 3.5B,B'). Together, these data indicate that Twist is involved in controlling the specification state necessary for a complete EMT process during PMC ingression.

In cell cultures, the Twist transcription factor has recently been shown to play an important role in the EMT process, which is associated with tumor metastasis, via transcriptional repression of E-cadherin (Yang et al. 2004). While an “in vivo” EMT was not observed in those cell cultures (a major drawback in cell cultures), but rather the overall phenotypes of the cells switched to mesenchymal, by which the authors interpreted the role of Twist as having been important in EMT. Here, our data provide in vivo evidence of Twist’s function on the EMT in sea urchin embryos. However, in Pmar1/TwiMASO co-injected experiments (Figure 3.7), the low penetrance of rescued embryos suggests that Twist’s role in ingression is much less important than the role of...
Snail (Wu and McClay, 2007). Intriguingly, several studies have shown that N-cadherin (a mesenchymal cell marker) is involved in tumor metastasis, motility and disruption of cell-cell adhesion (Islam et al. 1996; Nieman et al. 1999; Hazan et al. 2000; Li et al. 2001). Given the fact that Twist initiates the N-cadherin expression during the ventral furrow invagination in Drosophila (Oda et al. 1998), as well transcriptionally activating N-cadherin expression during metastasis (Alexander et al. 2006), it is possible that sea urchin Twist may engage a similar mechanism by expressing new types of adhesion molecules (not necessary N-cadherin per se) during PMC ingress to modulate EMT process, such as L1, as shown in breast carcinoma cells (Shtutman et al. 2006) or Cadherin-11 in Xenopus neural crest cells (Borchers et al. 2001). Although further tests are required for this hypothesis, the Twist family of transcription factors is added to the rapid-growing list of developmental regulators with a role in the EMT induction or tumor progression (Vernon and LaBonne 2004; Yang et al. 2004).

**Twist function is required for the maintaining the specification and differentiation of PMCs**

In Twi morphants, skeletal rudiments rarely form even after a prolonged culture period (Figure 3.4), which suggests that Twist may be required for the PMC differentiation process. The significant reduction of PMC-specific antibody (1d5) staining (Figure 3.6G-J) and differentiation gene expression (msp130, sm30, sm50; Table 2) in Twi morphants clearly supports this requirement.

In the micromere-PMC GRN, the downstream differentiation gene battery is under the control of the early PMC specification program, which includes alx1 and ets1. We further examined the regulatory relationships between these two genes and twist by WMISH (Figure 3.6 and Table 2). In Twi morphants, alx1 mRNA expression was down-regulated (although faint staining could be observed; Figure 3.6A,B), while the mRNA
expression level of ets1 was not significantly affected (Figure 3.6C,D). These data suggest that Twist may be essential for maintaining the specification of PMCs, since Twist mRNA starts to be expressed after the activation of alx1 (32–60 cell stage). The ERK signaling pathway has been shown to play a crucial role for the maintenance of PMC specification state probably via phosphorylation of one of the key target proteins, Ets1 (Fernandez-Serra et al. 2004; Rottinger et al. 2004). Blocking ERK signaling results in strong reduction of alx1 mRNA expression (Rottinger et al. 2004), which is similar to the phenotypes in Twi morphants. However, the unaffected ets1 expression in Twi morphants strongly rules against that twist being placed upstream of ets1, but twist may still act downstream of ERK signaling.

During Drosophila mesoderm specification, Twist responds to Dorsal and in turn activates Snail expression, which establishes the molecular cascade leading to ventral furrow invagination (Kosman et al. 1991; Ip et al. 1992). Recent studies also uncovered several downstream targets of Twist, which are independent of Snail (Seher et al. 2006; Kolsch et al. 2007); In sea urchins, the Snail transcription factor is required for PMC ingress (Wu and McClay, 2007) and PMC ingress is impaired in Twi morphants. We asked, therefore, if Twist is also upstream of Snail in sea urchin embryos. The WMISH of snail probe in Twi morphants showed a reduced staining signal (Figure 3.6F) and the expression level of snail mRNA in Twi morphants also showed a significant decrease compared to controls (Table 2). These data conclude that Twist may regulate Snail expression, either directly or indirectly through the connection to Alx1, although the exact regulatory hierarchy of these three genes awaits further detailed analyses.

It will be of interest to identify the upstream regulators of Twist, and preliminary QPCR results (data not shown) suggest that Alx1 and Snail, but not Ets1 may play a role in regulating Twist expression, although further experiments are needed for more conclusive data. If these data are confirmed, it means Twist is engaged in a feedback
loop with Alx1 (and also Snail), though detail of that notion have yet to be understood. Nonetheless, our data support that Twist acts as an ancillary regulator to maintain the steady network state of the PMC specification and differentiation.

**A role for Twist in skeletogenesis**

An aspect of Twist’s function in PMC development is its role in PMC fusion. Before the triradiate spicule rudiments are formed in the PMC ventrolateral clusters, the PMC filopodial protrusions fuse and join the cells into a syncytial network. In the absence of Twist, exogenous transplanted PMCs were unable to fuse with the endogenous PMCs to form the PMC syncytial ring, and also failed to express the MSP130 protein (Figure 3.8). The inability of these Twi-deficient PMCs to fuse likely results in the later phenotypes of skeletogenesis failure (Figure 3.3D,H-H’) though it could as well be the consequence of accumulated failures in morphogenesis. The Twi-morphant PMCs eventually begin to fuse and eventually at least partially stain with 1d5 (Figure 3.9B,C), but they remain disorganized and lack the skeletal rudiments (Figure 3.4). Interestingly, Twist has been shown to play roles in several types of fusion events in other systems, such as myoblast fusion in *Drosophila* via regulating *myoblasts incompetent/lame duck* (a Gli family of zinc-finger transcription factor) (Duan et al. 2001; Ruiz-Gomez et al. 2002), and mouse cranial suture fusion (Yoshida et al. 2005; Connerney et al. 2006).

Thus Twist seems to be expressed prior to ingression and each of the developmental processes examined from that point onward requires Twist activities. In the absence of Twist, the later processes of spiculogenesis and skeletal patterning suffer the most suggesting that there is an accumulated failure of morphogenetic events, which likely reflect an early (pre-ingression) role for Twist, followed by progressive losses of
necessary downstream components that eventually impair remaining aspects of the morphogenetic processes.

Twist plays a positive role in muscle formation in sea urchin embryos

Although the roles of twist gene in the embryogenesis has been well-documented in numerous studies (reviewed by Castanon and Baylies 2002), there is still a notable paradox about Twist’s function on muscle development in vertebrates and invertebrates (for reviews see Puri and Sartorelli 2000 and Baylies and Michelson 2001). After gastrulation in Drosophila embryos, the level of Twist is a critical determinant of mesodermal differentiation (Baylies and Bate 1996). Increasing Twist expression in mesodermal domains where Twist is usually low blocks formation of tissues such as the visceral mesoderm and leads to formation of ectopic body muscles. At the same time, expression of Twist at low levels interferes with somatic myogenesis but permits the development of other tissues (Baylies and Bate 1996). Taken together these experiments indicate that Twist promotes the formation of body muscle in Drosophila (Baylies et al. 1997; Baylies et al. 1998).

Twist appears to play an opposite role in vertebrate myogenesis, and mouse Twist has been shown to inhibit skeletal muscle formation in cell culture experiments (Spicer et al. 1996; Hamamori et al. 1997). Here, we asked whether sea urchin Twist positively or negatively regulates muscle development. Based on the myosin antibody staining (Figure 3.9A-C), we conclude that Twist acts as a positive regulator to muscle formation in sea urchin embryos, despite the fact that sea urchins are deuterostomes, like vertebrates. Further experiments are required to learn where Twist is involved in the specification trajectory leading to muscle. That specification begins with the Notch signaling pathway, as Notch and Delta have been shown to be essential for SMC
specification and subsequent muscle cell development in sea urchins (Sherwood and McClay 1999; Sweet et al. 2002). Since there is already a preliminary gene network for muscle cell precursors (Fernandez-Serra et al., 2004), it will be valuable to learn how Twist relates to that network state.

**Twist as an evolutionarily conserved modulator for mesoderm development**

Sea urchin Twist and other Twist family members in all metazoans examined to date have been demonstrated or implicated to have a common role in mesoderm specification and differentiation throughout evolution. Additionally, studies have indicated a conservation of certain downstream targets of Twist. For example, in C. elegans, the promoters of two genes, NK-class homeodomain (ceh-24) and FGFR-like genes (egl-15) contain the Twist E-box consensus sequence (Twist-binding site) and it has been demonstrated in vivo that CeTwist regulates these genes (Harfe and Fire, 1998). In Drosophila, activation of tinman and heartless, the homologues of the ceh-24 and egl-15 genes, respectively, requires Twist early in embryogenesis (Bodmer 1993; Shishido et al. 1993; Beiman et al. 1996; Gisselbrecht et al. 1996; Yin et al. 1997). While in sea urchins, an NK-class homeodomain gene, Hex (Meredith Howard-Ashby and Eric Davidson, personal communication) and FGFR1 (Lapraz et al. 2006) are both found to be expressed in PMCs and SMCs (among other territories). Given the role of Twist in mesoderm development, it is highly plausible that these two genes (among several others) are regulated by Twist, although detailed investigation is needed and will follow.

In conclusion, Twist exerts multiple functions during PMC development and participates in different network states of the endomesoderm GRN. Twist plays a role in the pre-EMT network state, in maintaining the PMC cell fates, in skeletogenesis, and also in muscle formation. Twist proteins can form homodimers or heterodimers with other bHLH proteins, and these dimers can have distinct regulatory functions (Castanon...
et al. 2001; Connerney et al. 2006). It will be of great value to identify the different dimer partners of Twist during sea urchin embryogenesis to further understand the biochemical function of Twist protein (as activator or repressor), and decipher the complex regulations of Twist in detail.
Chapter 4:
Expression Patterns of Six Transcription Regulators during Sea Urchin Embryogenesis
Introduction

The recently annotated sea urchin genome accelerates the discovery of new genes and holds the strong promise of mapping out the complete micromere-PMC GRN states. To build a more comprehensive picture of the complex EMT network in PMCs, we canvassed the PMC EST database and the expression data from published gene annotations, which then allow us to further cloned and identified several new PMC-enriched (or specifically expressed) genes in *L. variegatus* and examined the expression patterns of each gene, since the expression profiles of regulatory genes provide the essential preliminary information required for an understanding of their roles in developmental gene regulatory networks (GRNs). In our experience, the details of both spatial and temporal expression patterns are almost always relevant and significant for functional analysis. This chapter reports on the initial characterization of those genes as part of a project to build an ever more complete picture of the changing network states that drive mesoderm development.
Materials and Methods

Cloning

Based on the annotated gene list of Strongylocentrotus purpuratus genome and the available EST data, we designed primers to isolate corresponding genes from *L. variegatus* cDNA libraries. After amplification, the PCR products were cloned into either pGEMT-Easy (Promega) or pCS2 vectors. 5’RACE (with Ambion RLM-RACE) was performed to obtain the correct translation start sites, which will be helpful for designing morpholino antisense oligos for future functional analyses.

Alignment and phylogenetic analysis

Protein sequences for each gene analyzed were obtained by conceptual translation of the corresponding DNA coding sequence (open reading frame, ORF). Using the BLAST interface in the Metazome database (http://www.metazome.net), each protein sequence was used to search against the protein database (from ten sequenced animal genomes). The ClustalW alignment and the Neighbor-Joining tree were also constructed by utilizing the online tools provided from the website. The bootstrap value of each tree was omitted for presentation purposes.

Whole mount in situ hybridization (WMISH)

In situ hybridization was performed as previously described (Wu and McClay, 2007) with DIG-labeled RNA probes and BM purple substrate (Roche) for signal detection. Unless noted otherwise, most in situ probes correspond to the full-length open reading frame of each gene. Embryos before hatched blastula stage were omitted since we focused on genes expressed immediately before, during, and after PMC ingestion.
QPCR analysis provided the added information to determine whether the genes in question are expressed earlier than the time surrounding ingression.

Expression array

The temporal expression of each cloned gene was determined by utilizing the expression array database containing the sequences found as gene predictions within the *S. purpuratus* genome (Wei et al., 2006; http://urchin.nidcr.nih.gov/blast/exp.html). Although not a direct measurement of expression of *L. variegatus* genes, the expression profiles are generally similar between orthologues of the two species. Therefore, the expression profiles were downloaded from the database and expression charts were compiled for each gene. The expression level (abundance) of each gene was measured by the signal intensity of the array as artificial units (AU); where the mRNAs were present at ~1000–3000 molecules per embryo they give signals of around 500–1000 AU (Wei et al. 2006).
Results and Discussion

To assemble a complete EMT gene regulatory network state, we first need to identify all regulatory genes expressed in PMCs. Facilitated by the sequence data of the annotated *S. purpuratus* genome and the PMC EST database (Zhu et al. 2001), we have successfully cloned many full-length genes in *L. variegatus* to be added onto our gene list in the expanding EMT GRN. While this ambitious quest continues, we describe here the preliminary spatiotemporal expression profiles of six newly cloned transcription factors as a progress report for the initial phase of our ongoing project.

**Fos**

The Fos family of transcription factors includes c-Fos (the human homolog of the retroviral oncogene v-Fos), FosB, Fra-1 and Fra-2 as well as smaller FosB splice variants FosB2 and deltaFosB2 (reviewed in Milde-Langosch 2005). Partnered with Jun family members (c-Jun, JunB and JunD) they form the cluster of AP-1 proteins. The first AP-1 proteins (c-Jun and c-Fos) were found to have transforming activities in NIH3T3 rat fibroblasts (Miller et al. 1984), and soon after their discovery the AP-1 complex was then implicated in carcinogenesis. In contrast to Jun proteins, Fos family proteins are not able to form homodimers, but instead heterodimerize with Jun partners, giving rise to various trans-activating or trans-repressing protein complexes with diverse biochemical properties (Milde-Langosch 2005). In vitro experiments have shown that Jun–Fos heterodimers are more stable and have stronger DNA-binding activities than Jun–Jun homodimers (Halazonetis et al. 1988; Ryseck and Bravo 1991). Recent results suggest that some non-transforming Fos proteins, especially Fra-1 and Fra-2, may be involved in the progression of many tumor types. In addition, other recent studies in *C. elegans*...
identified *fos-1*, an ortholog of the vertebrate Fos gene family, as a crucial regulator of basement membrane removal during the anchor cell invasion (Sherwood et al. 2005).

**Expression pattern of sea urchin Fra**

In the course of searching for new transcription factors expressed by PMCs, we come across several EST clones containing different regions of a Fra (Fos-related antigen) gene homologue. The full-length open reading frame of *LvFra* gene was assembled and cloned through PCR and 5’RACE. The predicted amino acid sequence of LvFra shows a strong conservation within its basic leucine-zipper domain as compared to other Fos family genes, including Fra-1, Fra-2 and FosB (Figure 4.1A). Molecular phylogenetic analysis by the Neighbor-Joining tree supports the orthology of LvFra as a member of the Fos family of transcription factors (Figure 4.1B). The 5’RACE clone was used as a probe for WMISH. *LvFra* mRNA is expressed in PMCs only during the PMC ingression (Figure 4.2A-C). The expression array data shows that Fra transcripts accumulate soon after early blastula stage and continue at a relatively high level through pluteus larvae stage (Figure 4.2D).

The PMC-specific expression of *LvFra* occurs just at the time that PMCs begin to undergo an EMT and ingress into the blastocoel. It is very tempting to speculate that Fra plays an essential role in the EMT network state and is involved in regulating PMC ingression, and functional and perturbation studies are in line to test this hypothesis. The sea urchin Jun transcription factor is expressed in PMCs as well (Howard-Ashby et al. 2006), which further suggests a role for AP-1 complexes in regulating PMC development.
**Figure 4.1: Characterization of LvFra.** (A) ClustalW alignment of the protein sequence for LvFra with Fos family proteins, showing the most conserved region. Amino acids are color-coded with the default settings of the Clustal program. Similar amino acids at a given position in all proteins are shown by same color code. (B) Phylogenetic analysis showing that LvFra (in red) belongs to the Fos family and is close related to vertebrate Fra-2 and FosB genes. The neighbor-joining tree was constructed using the protein alignment in (A). Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Ce, *Caenorhabditis elegans*; Ci, *Ciona intestinalis*; Gg, *Gallus gallus*; Hs, *Homo sapien*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*.
Figure 4.1: Characterization of LvFra
Figure 4.2: Spatial and temporal expression of sea urchin Fra. (A-C) Whole mount in situ hybridization of LvFra. (A) As the PMCs starts to ingress, LvFra mRNA is exclusively expressed in the nascent PMCs. (B) LvFra is expressed in the ingressing PMCs at early mesenchyme blastula (MB) stage. (C) At MB stage, LvFra mRNA expression is clearly observed in PMCs. (D) Expression level, which is measured as signal intensity (shown in AU; see Methods for details) through developmental stages; the Fra expression begins around early blastula (EB) stage (15hr in S. purpuratus), and continues to increase at later stages. EG, early gastrula stage; LG, late gastrula stage; PL, pluteus larvae stage.
Figure 4.2: Spatial and temporal expression of sea urchin Fra
Mitf

Mitf (Microphthalmia-associated transcription factor) is a tissue-restricted, basic helix–loop–helix leucine zipper (b-HLH-Zip) transcription factor, which can form homo- and/or hetero-dimers with the HLH and Zip domains. The functions of Mitf are best represented by studies in the mouse. Mitf mutant mice have defects in melanocytes, the retinal-pigmented epithelium, mast cells and osteoclasts (Hodgkinson et al. 1993; Widlund and Fisher 2003; Steingrimsson et al. 2004). Additionally, germline heterozygous mutations of the MITF gene in humans are associated with the congenital pigmentation/deafness condition, called “Waardenburg Syndrome (WS) type IIA” (Hughes et al. 1994; Tassabehji et al. 1994; Nobukuni et al. 1996), in which affected individuals display variable degrees of pigmentation dilution and associated deafness due to melanocyte defects and defects in the inner ear (see review in Price and Fisher 2001).

The Mitf protein structure is highly conserved in both the basic domain and the helix-loop-helix domain between all species investigated to date (Steingrimsson et al. 2004). Further analysis has revealed conservation of Mitf gene function as well. In addition to the mouse, Mitf mutations have been identified in rat (Opdecamp et al. 1998; Weilbaecher et al. 1998), hamster (Hodgkinson et al. 1998; Graw et al. 2003), quail (Mochii et al. 1998), and zebrafish (Lister et al. 1999). In these species, as in mouse, the mutations also affect the development of neural crest–derived pigment cells. Mutations in nacre, one of the two zebrafish Mitf genes, affect only neural crest melanocytes, suggesting that the genome duplication event, which produced the two Mitf paralogues, was followed by the subsequent evolution of tissue-specific regulatory divergences (Lister et al. 1999; Lister et al. 2001). Moreover, preliminary genetic evidences in Drosophila show that the Mitf gene is expressed in the eye imaginal disc during embryogenesis. Targeted expression of wildtype or dominant-negative forms of
Mitf proteins results in opposite effects on the development of the eye disc region, especially affecting neuronal morphogenesis. This suggests that the role of Mitf in eye development may be partially conserved between *Drosophila* and vertebrates (Hallsson et al. 2004), which may suggest its original role before the separation of these lineages.

**Expression pattern of sea urchin Mitf**

The PCR-amplified open reading frame of *LvMitf* gene was cloned into the pCS2 expression vector. The HLH-Zip domain of the LvMitf protein is highly conserved when compared to those in other species (Figure 4.3A), and molecular phylogenetic analysis supports the notion that LvMitf belongs to the Mitf family of transcription factors (Figure 4.3B). WMISH, using a full-length probe, was performed to determine the spatial expression of *LvMitf* mRNA. As shown in Figure 4.4, the expression of *LvMitf* is restricted to PMCs starting from early mesenchyme blastula until mid-gastrula stage (Figure 4.4A-C). At late-gastrula stage, a small patch of the *LvMitf*-expressing cells can also be observed at the tip of the archenteron (Figure 4.4D); their location suggests they may be a subset of SMCs, although further confirmation will be required to say definitely. The temporal expression array data shows that *Mitf* expression increases rapidly after early blastula stage (Figure 4.4E) and peaks around early gastrula stage, which corresponds well to our WMISH data.

The SMC-localized expression of *LvMitf* is possibly consistent with the known role of *Mitf* in melanocytes, if the subset of SMCs with *LvMitf* expression is in fact the pigment cell lineage. However, the PMC-restricted expression of *LvMitf* is somewhat surprising, though interesting, since PMCs are devoted to forming only the larval skeleton and not for the production of pigment cells. However, recent studies implicate that *Mitf* is involved in the progression and the invasiveness of malignant melanoma (Garraway et al., 2005), which, if related, leads to the implication of a possible
**Figure 4.3: Characterization of LvMitf.** (A) ClustalW alignment of the protein sequence for LvMitf with representative Mitf genes in several species, showing the conserved b-HLH-Zip region. Amino acids are color-coded with the default setting of the Clustal program. Similar amino acids at a given position in all proteins are shown by same color code. (B) Phylogenetic analysis supporting that LvMitf (in red) belongs to the Mitf gene family. The neighbor-joining tree was constructed using the protein alignment in (A). Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Ce, *Caenorhabditis elegans*; Ci, *Ciona intestinalis*; Gg, *Gallus gallus*; Hs, *Homo sapien*; Lv, *Lytechinus variegatus*; Mm, *Mus musculus*; Xt, *Xenopus tropicalis*. 
Figure 4.3: Characterization of LvMitf
Figure 4.4: Spatial and temporal expression of sea urchin Mitf. (A-D) Whole mount in situ hybridization of LvMitf. (A-C) LvMitf is expressed in PMCs only from early MB stage (A) to mid-gastrula (MG) stage (C). (D) At late gastrula (LG) stage, a small patch of Mitf-expressing cells can be observed at the tip of the archenteron (arrow), along with the persistent PMC expression. (E) Expression level throughout development; Mitf expression accumulates rapidly after EB stage (15hr in S. purpuratus), and then climaxes at early gastrula (EG) stage.
Figure 4.4: Spatial and temporal expression of sea urchin Mitf
functional role of sea urchin *Mitf* in regulating PMC ingress. Also, since *Mitf* is expressed in vertebrate neural crest cells, there could also be a functional relationship since neural crest cells are known to go through both an epithelial to mesenchymal transition and then derived pigment cells invade the epithelium.

**FoxO**

FoxO transcription factors belong to the large Forkhead (or winged-helix) family of proteins, a family of transcriptional regulators characterized by a highly conserved DNA-binding domain termed the “Forkhead box” (Kaestner et al. 2000). The Forkhead family is present in all eukaryotes. In humans, the Forkhead family is comprised of 39 distinct members, which have been divided into 19 subgroups (Fox A to S)(Mazet et al. 2003). Among these subgroups, some are constitutively expressed across a broad spectrum of tissue types while others are expressed in a restricted spatiotemporal manner. In particular, FoxO transcription factors have been implicated to be at the interface of crucial cellular processes, orchestrating programs of gene expression that regulate apoptosis, cell-cycle progression, longevity, and oxidative stress resistance (see in-depth reviews in Accili and Arden 2004; Greer and Brunet 2005; Arden 2006). In sea urchins, there have been 22 Fox family genes annotated from the *S. purpuratus* genome (Tu et al. 2006), and one of which, SpFoxA, is involved in endoderm specification (Oliveri et al., 2006).

**Expression pattern of sea urchin FoxO**

The open reading frame of the *LvFoxO* gene was PCR-amplified from a *L. variegatus* gastrula stage cDNA library and the PCR product was subsequently TA-cloned. When aligned with ClustalW, the predicted *LvFoxO* amino acid sequence and SpFoxO protein (GenBank Accession Number ABB89484) share an amino acid identity of almost 100%,
with only few substitutions (data not shown). The spatial expression pattern of LvFoxO was determined by WMISH with a probe containing full length ORF (Figure 4.5A-C). The *LvFoxO* staining can be detected in the PMCs as they begin to ingress (Figure 4.5A-B), continuing to be expressed in these cells later in development, with slightly stronger signals in the ventrolateral PMC clusters (Figure 4.5C). The temporal expression pattern of *FoxO* mRNA shows that the transcripts accumulate starting from early blastula stage, peaking at the late gastrula stage (Figure 4.5D).

Intriguingly, the FoxO transcription factors have been shown to function in other systems as transcriptional mediator, directly downstream of the PI3K/PKB signaling pathway, and initiated by insulin-like factors (see Greer and Brunet 2005; Kenyon 2005) for detailed reviews). It is known that in the sea urchin the inhibition of P13K activity blocks skeletogenesis (Bradham et al. 2004), and the PMC expression of FoxO makes it a highly plausible candidate as the downstream target of the P13K signaling required in the spicule formation.

**SoxC**

The Sox family of transcription factors was first identified in mammals in 1990 based on the conservation of the HMG box of the mammalian testis-determining factor SRY gene (Gubbay et al. 1990). Subsequently, Sox family transcription factors have been found in all metazoans, where they play many key roles during embryonic development. The mammalian Sox family comprises some 20 genes and can be divided into 10 subgroups on the basis of sequence similarity and genomic organization (Bowles et al. 2000). Sox proteins bind sequence-specifically to DNA by means of a high-mobility group (HMG) domain, allowing them to function as transcriptional regulators. Mutations in several of Sox genes have been shown to result in developmental anomalies.
Figure 4.5: Spatial and temporal expression of sea urchin FoxO. (A-C) Whole mount in situ hybridization of *LvFoxO*. (A-B) *LvFoxO* is expressed in PMCs at early and late MB stages. (C) At LG stage, *LvFoxO* is still expressed in PMCs, especially concentrated in the ventrolateral PMC clusters. (D) Expression level throughout development; *FoxO* expression steadily increases from early stages and reaches to a peak at LG stage.
Figure 4.5: Spatial and temporal expression of sea urchin FoxO
In humans, for example, SOX9 mutations cause skeletal dysmorphology and the sex reversal syndrome campomelic dysplasia (whereas SRY mutations result in sex reversal and gonadal dysgenesis), while SOX10 mutations underlie the neurocristopathy syndromes Waardenburg–Hirschsprung and Yemenite deaf-blind hypopigmentation (reviewed in Wilson and Koopman 2002). The SoxC subgroup comprises vertebrate Sox4 and Sox11 genes, and both *Sox4* and *Sox11* mutant mice exhibit defects in the neural crest derivatives (Hong and Saint-Jeannet 2005).

**Expression pattern of sea urchin SoxC**

The full-length ORF of the *LvSoxC* gene was PCR-cloned into a TA-cloning vector. As expected, the SOX-TCF-HMG domain of LvSoxC protein is highly conserved with other SoxC subgroup members (Figure 4.6A), and the phylogenetic analysis clearly supports the orthology of LvSoxC (Figure 4.6B). The vertebrate Sox4 genes, also in the SoxC subgroup, are not included here for simplification of the analysis. The spatial expression of *LvSoxC* was determined by WMISH with a full-length *LvSoxC* probe (Figure 4.7). *LvSoxC* is expressed in the presumptive PMC territory at the vegetal plate before the ingression begins (Figure 4.7A). When PMCs start to ingress, the *LvSoxC* mRNA expression remains at the vegetal plate (Figure 4.7B), in cells that will become the future SMCs (and later will ingress), while the PMC expression of *LvSoxC* diminishes progressively after they ingress (Figure 4.7C). The temporal expression array data shows a steady increase of SoxC expression from early blastula to pluteus larvae stage (Figure 4.7E). The transient expression of *LvSoxC* in the micromere descendents suggests the possibility that it functions in early PMC specification and pre-EMT network state, which is currently under investigation.
Figure 4.6: Characterization of LvSoxC (A) ClustalW alignment of the protein sequence for LvSoxC with representative SoxC subgroup genes from several species, showing the conserved SOX-TCF-HMG domain. Amino acids are color-coded with the default setting of the Clustal program. Similar amino acids at a given position in all proteins are shown by same color code. (B) Phylogenetic analysis supporting that LvSoxC (in red) is closely related to vertebrate Sox11 genes (belongs to SoxC subgroup). The neighbor-joining tree was constructed by using the protein alignment in (A). Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Ce, *Caenorhabditis elegans*; Ci, *Ciona intestinalis*; Gg, *Gallus gallus*; Hs, *Homo sapien*; Lv, *Lytechinus variegatus*; Mm, *Mus musculus*; Xt, *Xenopus tropicalis*.
Figure 4.6: Characterization of LvSoxC
Figure 4.7: Spatial and temporal expression of sea urchin SoxC. (A-C) Whole mount in situ hybridization of LvSoxC. (A) LvSoxC is expressed in the presumptive PMC territory at hatched blastula stage. (B-C) As PMC ingestion begins, the expression of LvSoxC is gradually downregulated in PMCs, but remains at the vegetal plate where become the future SMCs (C). (D) Expression level throughout development; the SoxC expression steadily increases as the embryogenesis proceeds.
Figure 4.7: Spatial and temporal expression of sea urchin SoxC
The T-box gene family, which is defined by a common DNA-binding domain known as the T-box, is evolutionarily ancient and probably arose in the common ancestor of metazoan organisms (Agulnik et al. 1996). This family is present in all metazoans and consists of 17 genes in mammals organized into 5 subfamilies. T-box genes first came to the attention of geneticists in 1927 with the discovery of an interesting mutation, Brachyury (or T, for short-tail), which caused truncated tails in mice (Dobrovolskaia-Zavadskaia, 1927) and was later cloned in 1990 (Herrmann et al. 1990). T-box genes are expressed throughout embryonic development in dynamic patterns with both unique and overlapping areas of expression. In recent years, both spontaneous and induced mutations in T-box genes have demonstrated that these genes are important developmental regulators, controlling patterning in a wide range of tissues and organs, as well as contributing to several human congenital syndromes (see review in Papaioannou 2001; Naiche et al. 2005).

Expression pattern of sea urchin Tbx20

After being PCR-amplified, the full-length *LvTbx20* gene was TA-cloned. The ClustalW alignment and the phylogenetic analyses strongly support *LvTbx20* being one of the Tbx20 family members, including *Drosophila* H15 and *C. elegans* mab-9 (Figure 4.8A-B). WMISH (with a N-terminal probe) shows *LvTbx20* is expressed in PMCs during PMC ingression, and then also in SMCs at early gastrula stage (Figure 4.9A-C). The expression array detects a very high abundance of *Tbx20* transcripts in 2-cell stage, but then the expression level plummets rapidly to almost none by early gastrula stage (Figure 4.9D). Nonetheless, we can detect signals at early gastrula stage in *L. variegatus* by WMISH, and this discrepancy is still under investigation. Interestingly, Tbx20 genes have been recognized as important regulators in cardiac development and heart
Figure 4.8: Characterization of LvTbx20 (A) ClustalW alignment of the protein sequence of LvTbx20 with representative Tbx20-related genes of several species, showing the conserved T-box domain. Amino acids are color-coded with default setting of the Clustal program. Similar amino acids at a given position in all proteins are shown by same color code. (B) Phylogenetic analysis supporting LvTbx20 (in red) placement in the Tbx20 subfamily of T-box genes. The neighbor-joining tree was constructed by using the protein alignment in (A). Dm, Drosophila melanogaster; Dr, Danio rerio; Ce, Caenorhabditis elegans; Ci, Ciona intestinalis; Gg, Gallus gallus; Hs, Homo sapien; Lv, Lytechinus variegatus; Mm, Mus musculus; Xt, Xenopus tropicalis.
Figure 4.8: Characterization of LvTbx20
Figure 4.9: Spatial and temporal expression of sea urchin Tbx20. (A-C) Whole mount in situ hybridization of LvTbx20. (A-B) LvTbx20 is expressed in PMCs during PMC ingression. (C) At EG stage, LvTbx20 is expressed in both PMCs and SMCs. (D) Expression level through developmental stages; The Tbx20 expression declines rapidly after the 2-cell stage, indicating a predominantly maternal expression pattern.
Figure 4.9: Spatial and temporal expression of sea urchin Tbx20
formation (Plageman and Yutzey 2005; Shelton and Yutzey 2007); however, as the heart is absent in sea urchins, the molecular function of sea urchin Tbx20 may be used instead to participate in regulation of early mesoderm development as suggested by the WMISH experiments.

Alx4

Alx4 encodes a Paired-class homeodomain transcription factor that evolved as a pleiotropic developmental regulator in vertebrates (Meijlink et al. 1999). Its multiple roles in skeletal patterning, differentiation, and growth are best revealed in Alx4−/− mice, which display craniofacial (including skull defects), axial and appendicular abnormalities as part of their complex phenotypes (Qu et al. 1997; Antonopoulou et al. 2004). In humans, heterozygous loss-of-function mutations of ALX4 can result in skull vault defects, characteristically in the form of enlarged parietal foramina (PFM, (Mavrogiannis et al., 2001; Mavrogiannis et al., 2006), and large deletions encompassing ALX4 define the proximal 11p deletion syndrome (P11pDS; also known as Potocki-Shaffer syndrome)(Bartsch et al. 1996; Wakui et al. 2005).

Expression pattern of sea urchin Alx4

The full-length LvAlx4 clone was isolated from a cDNA library by PCR using the sequence information of the annotated SpAlx4 gene (SPU_022816). The amino acid sequence of LvAlx4 is highly similar to that of LvAlx1 (data not shown), which has been shown to be essential for PMC specification (Ettensohn et al., 2002). The LvAlx4 also contains a conserved C-terminal OAR (otp, aristalless, Rx) domain as found in the LvAlx1 protein; in fact many Paired-class homeodomain proteins, including members of the Cart1/Alx3/Alx4 subfamily, have an OAR domain at the C terminus (Galliot et al. 1999). WMISH using a full-length probe shows that LvAlx4 mRNA is expressed in
PMCs at the mesenchyme blastula and early gastrula stages (Figure 4.10A-B), and then is further restricted to the PMC ventrolateral clusters at the late gastrula/prism stage (Figure 4.10C). As shown in Figure 4.10D, the expression of Alx4 peaks around early gastrula stage and declines afterwards. It is reasonable to imagine a function for Alx4 similar to that of Alx1 in PMC specification, but as the expression of Alx4 occurs later, it is possible that Alx4 plays a role in regulating PMC differentiation and skeletogenesis.
Figure 4.10: Spatial and temporal expression of sea urchin Alx4. (A-C) Whole mount in situ hybridization of *LvAlx4*. (A-B) *LvAlx4* is expressed in PMCs from MB to EG stages. (C) The vegetal view of an LG stage embryo. *LvAlx4* mRNA is concentrated in the ventrolateral PMC clusters. (D) Expression level throughout development; the *Alx4* expression peaks around EG stage and declines afterwards.
Figure 4.10: Spatial and temporal expression of sea urchin Alx4
Conclusion

The sea urchin genome contains invaluable sequence information for use in identifying additional transcription factors or signaling molecules expressed during embryogenesis. This will allow us to progressively assemble the EMT network states and PMC GRN in a much more efficient and complete fashion. Here, we take advantage of the gene annotation to isolate several PMC genes from *L. variegatus* and investigate their spatial and temporal expression patterns, as a preliminary exercise that will lead to a functional understanding of these transcription factors. Positioning these new genes into the sea urchin PMC GRN by further in-depth perturbation studies will help us elucidate the dynamic regulatory network state *before* and *after* PMC ingression, which might contribute to our understanding of the molecular mechanisms involved in tumor progression and metastasis.
Chapter 5:

Conclusions
Summary

This dissertation focuses on molecular mechanisms and gene regulatory networks of PMC ingression by investigating the functions of two transcription factors, Snail and Twist, in regulating EMT during sea urchin embryogenesis. Furthermore, this work also examines the spatiotemporal expression patterns of several newly isolated PMC genes in *L. variegatus*, which hold promise for construction of a more complete EMT network in PMCs. These key findings described in previous chapters are summarized in brief here.

Snail is required for PMC ingression

We identify a sea urchin orthologue of the Snail transcription factor (Chapter 2), and show it to be expressed just before the PMCs begin to ingress into blastocoel. Functional knockdown analyses of Snail in whole embryos and chimeras demonstrate that Snail is required in micromeres for PMC ingression. Perturbation studies also position Snail in the sea urchin micromere-PMC gene regulatory network, downstream of Pmar1 and Alx1, and upstream of several PMC differentiation genes. Taken together, our findings indicate that Snail is essential in PMCs for the ingestion.

Snail represses cadherin expression and regulates cadherin endocytosis

We show that Snail represses cadherin expression in PMCs, a repression that appears evolutionarily conserved throughout the animal kingdom. Further, Snail expression is required for endocytosis of cadherin, a cellular activity that accompanies PMC ingression. Along with the EMT defect in Sna morphants, we conclude that Snail regulates EMT in part through its repression of cadherin expression during PMC ingression, and in part through its function in the endocytosis that facilitates the conversion from an epithelial cell to a mesenchyme cell.
Twist is essential for proper PMC ingression, PMC fusion, and skeletogenesis

Twist, a mesoderm specification gene, has been implicated in controlling the metastasis of cancer cells (Yang et al., 2004). We identify the sea urchin orthologue of Twist, and characterize its function with the focus of EMT processes and mesoderm development (Chapter 3). Phenotypes observed in Twist-deficient embryos by morpholino knockdown indicate that Twist is required for proper EMT to occur in PMCs, and skeletogenesis as the spicules are rarely formed. Micromere transplant experiments show that Twist also plays an important role in PMC fusion. Moreover, another defect in Twist morphants is the loss of muscle formation, which is a conserved role for Twist family genes. Current efforts are to place Twist in the micromere-PMC network, and preliminary results indicate that Twist is likely to be responsible for the maintenance of PMC specification state.

The construction of a complete EMT gene regulatory network

Studying gene regulatory networks enables the understanding of the mechanism underlying the developmental process at the most fundamental level. To build a more comprehensive picture of EMT network state operating in PMCs at the time of ingression, we further clone and identify several new PMC genes with genomic information from the sea urchin genome, and report the initial characterization and the expression patterns of each gene. Many of these transcription factors, including Fos and Mitf, are particularly intriguing since they have been implicated in cell movement, neural crest cell formation, and cancer metastasis (Chapter 4). Future experiments will focus on elucidating the roles of these genes in PMC ingression, as well as their connections to known PMC specification genes, such as Alx1 and Ets1, and PMC differentiation genes.
This would allow us to decipher the complex interconnections that regulate the EMT regulatory network state.
Future Directions

**Complete portrait of EMT gene regulatory network in PMCs**

Clearly, our ultimate goal is to fully understand the mechanisms underlying the EMT process occurred during PMC ingression by studying PMC gene regulatory network. In order to do so we not only have to identify the full complement of regulatory genes involved in this subnetwork, but also to map out with precision the causal regulatory relationships among/ between them and downstream differentiation genes. The annotated sea urchin genome accelerates the discovery of new genes and holds strong promise of mapping out the complete micromere-PMC GRN (preliminary results in Chapter 4). Combining the perturbation studies and already available microarray resources, we can rapidly construct an increasingly complete EMT network in PMCs, which will be valuable for us not only to unravel the complex coordination of dynamic EMT regulatory network states, but also contribute useful insight into the molecular strategies that regulate EMT events in other organisms. Furthermore, comparing the EMT gene networks, which seem to be fundamentally conserved, between different cells in different organisms (e.g. neural crest cells) will help us (and hopefully will help those who work on other systems that exhibit EMT), to discover the underlying core elements of regulating cell movements.

**The regulation of cadherin endocytosis**

During PMC ingestion, cadherin proteins are rapidly endocytosed as PMCs enter the blastocoel (Miller and McClay 1997). In Chapter 2, we show that Snail plays a positive role in regulating the endocytosis of cadherins, but the exact cellular mechanism is still unclear. Attenuation of cell-cell adhesion is a key component of an EMT process, and cadherin endocytosis is an efficient strategy. While transcriptional repression can
regulate cadherin expression level, a precise protein turnover is also required to be effective in a time-dependent event. When cadherin transcription ceases in the micromere progeny of the sea urchin embryo, the EMT follows shortly thereafter, at a time when there is still an abundant reserve of cadherin proteins on the membrane of the future PMCs. Thus, for cadherin proteins to be eliminated acutely during the EMT, an endocytosis followed by degradation seems the most efficient mechanism.

It is possible that this endocytic event is clathrin-mediated or caveolin-mediated, and with suitable reagents and possible candidate genes (like caveolin-1), we can try to pinpoint the molecular requirements for this dramatic and exciting cellular event. By understanding the regulation of cadherin endocytosis, we may be able to link the EMT regulatory network to the cellular responses during PMC ingestion, which potentially would allow us to explain the PMC ingestion from an innovative and broad perspective. The fact that Snail is involved in this process provides a connection between the EMT network and the regulation of cadherin endocytosis.

**EMT gene network and cell lineage conversion**

In the sea urchin embryo, loss of PMCs by microsurgical removal or ablation of the micromeres at the 16-cell stage can be compensated for later by SMCs, some of which will trans fate and differentiate into skeletogenic mesenchyme (Ettensohn and McClay 1988; Ettensohn 1992). This fascinating phenomenon of cell lineage conversion serves a perfect example to demonstrate the regulative abilities of sea urchin embryos, which also provides us a great model to study the mechanisms of developmental plasticity. Previous studies showed that Alx1 and ERK activity is essential for the occurrence of this conversion (Ettensohn et al., 2003; Rottinger et al., 2004). It would be of great interest to investigate the expression of Snail and Twist, as well as their roles, during the transfating of SMCs. Based on the importance of Snail and Twist in regulating
EMT during PMC ingression, we hypothesize that both Snail and Twist will play crucial roles in this SMC-PMC transfating process. Interestingly, Snail is also involved in the EMT of SMCs (chapter 2), so the regulation of Snail may become more complex during the cell lineage conversion. However, to our surprise, Pmar1 is not expressed during the conversion (Charles Ettenshon, personal communication), which suggests that the re-initiation of PMC GRN is likely independent of the control of Pmar1. Further analyses of the regulation of the PMC network state transition, especially the EMT genes, during the SMC transfating will shed some light on regulative deployment of the PMC GRN.

Specifically, the very next experiment to figure out the response(s) of the PMC GRN during this cell conversion will be simply doing a careful temporal and spatial analysis of PMC genes for a micromereless embryo, in which SMCs will transfate in order to compensate the loss of PMCs. We will then ask if the PMC genes will be re-expressed in a same order sequentially or re-programmed to appear in a different order under this circumstance.

The regulation and evolution of EMT gene network and PMC GRN

The calcareous larval skeleton of euechinoid sea urchins (e.g. *L. variegatus*) is synthesized by primary mesenchyme cells which ingress prior to gastrulation. In contrast, in the cidaroid *Eucidaris tribuloides*, no mesenchyme cells ingress prior to archenteron invagination (Tennent, 1914, 1922; Schroeder, 1981; Wray and McClay, 1988). Despite their lack of early-ingressed mesenchyme, *Eucidaris* larvae nonetheless contain skeletons. This particularly interesting developmental difference between cidaroids and euechinoids is likely due to a heterochrony in the ingestion time of spicule-forming cell (PMC) (Wray and McClay, 1988). It would be very interesting to examine the expression patterns of Snail and Twist in *Eucidaris*, which may help us to understand the molecular modification underlying this heterochronic event. Moreover,
analysis of cis-regulatory elements of EMT genes in Eucidaris could provide insights into the evolutionary processes that affect the regulation of EMT gene network state at the DNA level.

Other echinoderms, like starfish (asteroids), do not produce a larval skeleton during embryogenesis, which is likely the pleisiomorphic state, because a skeletogenic micromere lineage in the embryo is a relatively recent echinoid invention (Wray and McClay, 1988). It is possible that the EMT genes in starfishes (likely expressed in mesoderm) are co-opted into the PMC GRN in sea urchins, and a comparative analysis of expression patterns of EMT genes in both classes of echinoderms will reveal the molecular basis of this evolutionary novelty in echinoids. Additionally, SMCs also undergo ingression, though later than PMCs. PMCs are thought to be a subset of mesenchyme which precociously ingress. SMCs (the remaining mesenchyme) may employ the same process for ingestion as PMCs, so it would be valuable to compare the two networks controlling ingression to learn how PMCs evolved the precocious onset mechanism.
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