The control of foxN2/3 expression in sea urchin embryos and its function in the skeletogenic gene regulatory network

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SUMMARY
Early development requires well-organized temporal and spatial regulation of transcription factors that are assembled into gene regulatory networks (GRNs). In the sea urchin, an endomesoderm GRN model explains much of the specification in the endoderm and mesoderm prior to gastrulation, yet some GRN connections remain incomplete. Here, we characterize FoxN2/3 in the primary mesenchyme cell (PMC) GRN state. Expression of foxN2/3 mRNA begins in micromeres at the hatched blastula stage and then is lost from micromeres at the mesenchyme blastula stage. foxN2/3 expression then shifts to the non-skeletogenic mesoderm and, later, to the endoderm. Here, we show that Pmar1, Ets1 and Tbr are necessary for activation of foxN2/3 in micromeres. The later endomesoderm expression of foxN2/3 is independent of the earlier expression of foxN2/3 in micromeres and is independent of signals from PMCs. FoxN2/3 is necessary for several steps in the formation of the larval skeleton. Early expression of genes for the skeletal matrix is dependent on FoxN2/3, but only until the mesenchyme blastula stage as foxN2/3 mRNA disappears from PMCs at that time and we assume that the protein is not abnormally long-lived. Knockdown of FoxN2/3 inhibits normal PMC ingression and foxN2/3 morphant PMCs do not organize in the blastocoel and fail to join the PMC syncytium. In addition, without FoxN2/3, the PMCs fail to repress the transfating of other mesodermal cells into the skeletogenic lineage. Thus, FoxN2/3 is necessary for normal ingression, for expression of several skeletal matrix genes, for preventing transfating and for fusion of the PMC syncytium.

KEY WORDS: Gene regulatory network, Sea urchin, Forkhead transcription factor, Specification

INTRODUCTION
The sea urchin micromere gene regulatory network (GRN) is one of the best understood of all metazoan embryonic networks (Ettensohn et al., 2007; Oliveri et al., 2002; Oliveri et al., 2003; Oliveri et al., 2008). It specifies the large micromeres toward a skeletogenic fate. Micromeres initiate specification earlier than most cells of the embryo and their GRN operates in a largely cell-autonomous manner. At the early mesenchyme blastula stage, micromeres ingress into the blastocoel by an epithelial-mesenchymal transition (EMT) and differentiate into PMCs. They migrate inside the blastocoel with predictable behavior (Malinda and Ettensohn, 1994; Malinda et al., 1995; Peterson and McClay, 2003) and eventually form a characteristic ring surrounding the archenteron with two ventrolateral clusters gathering to initiate skeletogenesis. The ring of PMCs fuses to form a syncytium before it produces the larval skeleton. The micromere GRN model attempts to explain each step of micromere specification from the birth of micromeres at the fourth cleavage to ingestion and begins to explain parts of the terminal differentiation into skeletogenic cells.

Micromere specification is launched when β-catenin enters micromere nuclei starting at the fourth cleavage, and, with maternal Otx, activates pmar1, which is a transcriptional repressor and one of the earliest activated PMC-specific transcription factors (Logan et al., 1999; Oliveri et al., 2003). Pmar1 represses a ubiquitous repressor, hesC, thereby providing a double-repression gate that activates micromere-specific genes (Revilla-i-Domingo et al., 2007). Recent data suggest a delay in the repression of hesC, indicating that at least some of the downstream transcription factors are activated independently of the Pmar1/ hesC gate (Sharma and Ettensohn, 2010). As a consequence of these early events, transcription factors including Alx1, Ets1 and Tbr are activated (Croce et al., 2001; Ettensohn et al., 2003; Fuchikami et al., 2002; Kurokawa et al., 1999; Revilla-i-Domingo et al., 2007). Knockdown (KD) of early micromere transcription factors (TFs), such as alx1, results in impaired PMC ingression, repressed skeletogenic gene expression and malformed larval skeletons. Perturbation of these early-activated TFs at the top of the GRN thus causes catastrophic failures, whereas TFs activated at later stages of specification produce more specific responses if knocked down. For example, Snail and Twist, which are expressed in micromeres after the hatched blastula stage, play crucial roles during PMC ingression (Wu and McClay, 2007; Wu et al., 2008). Rather than regulating ingression, TFs like Hex or Tgif instead control skeletogenesis and biomineralization through activation of skeletogenic genes such as msp130, sm30 and sm50 (Oliveri et al., 2008). Thus, understanding the specification and differentiation of PMCs requires one to understand the sequential construction of the PMC network.

Despite an advanced level of understanding of the micromere GRN, there are a number of questions that remain to be answered. For example, the current micromere GRN model includes foxN2/3, although the only input modeled is β-catenin, and the only output is neutralized, a regulator of delta signaling (http://supp.caltech.edu/endomes/; August 12, 2010). As foxN2/3 is expressed many hours after β-catenin enters the nuclei of micromeres, we suspected that inputs to foxN2/3 were incomplete. Also, the FoxN2/3-neutralized relationship was based on a
quantitative polymerase chain reaction (QPCR) survey reported a number of years ago, and no detailed studies of foxN2/3 had been done beyond that survey. For that reason, we decided to investigate how FoxN2/3 works in the micromere GRN in greater detail.

Fox genes are members of the Forkhead TF family, which is characterized by a highly conserved forkhead-winged-helix motif, a helix-turn-helix motif consisting of three helices and two large loops (or wings) with DNA binding activity (Clark et al., 1993; Kaestner et al., 2000; Kaufmann et al., 1994; Li and Tucker, 1993). Compared with other Fox TF subfamilies, the FoxN subfamily members were discovered more recently. In mammals, the FoxN subfamily has six members: FoxN1 (Nehls et al., 1994), FoxN2 (human T-cell leukemia virus enhancer factor, HTLF) (Li et al., 1992), FoxN3 (checkpoint suppressor, CHES1) (Pati et al., 1997; Scott and Plon, 2003), FoxN4 (Gouge et al., 2001), FoxN5 (FoxR1) (Kato, 2004a and foxN6 (FoxR2) (Kato, 2004b), FoxN1 is widely known because a FoxN1 mutation produces the immune-deficient nude mice. In sea urchin, 22 fox genes were identified from the Strongylocentrotus purpuratus genome by searching for the conserved forkhead motif, and two members of the FoxN subfamily were found: FoxN1/4 and FoxN2/3 (Tu et al., 2006).

In this study, FoxN2/3 was cloned from Lytechinus variegatus and its regulation and function were investigated. We found that FoxN2/3 expression in micromeres is regulated by Pmar1, Ets1 and Tbr; foxN2/3 is also expressed in the non-skeletal mesoderm territory and later in the endoderm in an expanding torus pattern. However, that expression torus of foxN2/3 expression is not dependent on the Blimp1-Wnt-Otx subcircuit that was recently published for several other endomesoderm genes (Smith et al., 2008; Smith et al., 2007). Micromere progeny require FoxN2/3 to ingress in a timely manner and without FoxN2/3 PMCs fail to join the syncytium, and fail to block transiting. In addition, FoxN2/3 is necessary for activation of the early expression of many PMC specific genes.

MATERIALS AND METHODS

Animals

Lytechinus variegatus adults were obtained from Sea Life (Tavernier, FL, USA) or from Maria Wise (Duke University Marine Laboratory at Beaufort, NC, USA). Gametes were obtained by 0.5M KCl injection. After fertilization, embryos were cultured in artificial sea water at 23°C or room temperature.

Morpholino antisense oligonucleotides (MASO), mRNA injections and drug treatments

Two LyfoxN2/3-specific MASOs were obtained from Gene Tools (foxN2/3 MASO 1: TTCGGCTTTGCGATTTAGGATGCGATG; foxN2/3 MASO 2: AGATTTTGGCCCTGTATGCGTCC). foxN2/3 MASO 1 was injected at 0.5 mM or 0.7 mM and foxN2/3 MASO 2 was injected at 1 mM. Both caused identical phenotypes and identical gene knockdown consequences. The alx1b MASO was injected at 1 mM (Ettensonhoh et al., 2003). The blimp1b MASO (CAGAGAAATGAGAAGAATGCCTCC or CCGTTTCTGTAGGAAACACACAGC) was injected at 1 mM and a tbr MASO (ATCTCGAATCTTCGAGGCCT) was injected at 0.5 mM. Standard control MASO (CCTCTTACCAGTTCAATTTATA) (Gene Tools) was injected at 0.5-1 mM according to the experimental MASO concentration. Controls for novel morpholinos are presented in the text and supplementary figures. foxN2/3 CDS was cloned to pCS2 vector and transcribed using the mMessage Machine Kit (Ambion), pmar1, Dn-Notch, and Dn-Ets1 mRNA were transcribed as described previously (Sharma and Ettensonhoh, 2010; Sherwood and McClay, 1999; Wu and McClay, 2007). U0126 (Promega) was dissolved in DMSO and added to cultures from early cleavage stages at 10-30 μM.

Micromere-transplantation experiments

Micromere transplantsations were performed at the 16-cell or 32-cell stage. Detailed procedures were followed as described previously (Logan et al., 1999).

QPCR

Total RNA was isolated using Trizol (Invitrogen) or the RNeasy Mini Kit (Qiagen). Reverse transcription was performed with a Taqman RT-PCR kit (Applied Biosystems) or iScript cDNA synthesis kit (BioRad). QPCRs were performed using Mastercycler (Eppendorf) with Power SYBR Green PCR Master Mix (Applied Biosystems). The CT (crossing point threshold) was normalized using ubiquitin.

Immunostaining

Embryos were fixed in ice cold methanol for 1 minute, then washed four times in PBST, blocked in 4% normal sheep serum (NSS) in PBST for 30 minutes and incubated in 1d5 mouse antibody (anti MSP-130) (1:200) in 4% NSS PBST overnight at 4°C. After washing four times in 4% NSS PBST, samples were incubated in Cy2-conjugated secondary antibodies (Jackson Laboratories) (1:200) for 30 minutes, and washed in 4% NSS PBST four times and imaged in 50% glycerol and 50% PBST using Zeiss LSM510 confocal microscope.

In situ hybridization

Chromogenic in situ hybridization was performed using standard methods, with DIG-labeled RNA probes and NBT/BCIP (Roche). For double fluorescence in situ hybridization, digoxigenin-11-UTP and fluorescein-12-UTP (Roche) tagged probes were used at 1 ng/μl and detected with Cy3-tetramide and fluorescein-tetramide reagents using the TSA-plus Kit (Perkin Elmer). Hybridization and washing were carried out at 65°C. All samples were imaged with a Zeiss Axiospland2 microscope.

RESULTS

Identification and cloning of L. variegatus foxN2/3

A fragment of LvfoxN2/3 was cloned by PCR from a cDNA pool of L. variegatus, using primers designed from the S. purpuratus sequence. The 5' UTR was obtained by RACE PCR. The nucleotide sequences of L. variegatus and S. purpuratus were highly similar (87%) except the first exon, which covers part of the 5’ UTR.

Based on the expected open reading frame from the sequence data, LvfoxN2/3 encodes a 534 amino acid protein with a forkhead domain located from residue 125 to 212 (http://www.expasy.ch/prosite/; accession number HQ127623). The overall similarity of the amino acid sequences of L. variegatus foxN2/3 and S. purpuratus foxN2/3 was 93%, with 100% similarity in the forkhead domain (see Fig. S1A in the supplementary material). Phylogenetic analysis showed that LvfoxN2/3 clusters with foxN2 and foxN3 of Chordates; the amino acid sequence of the forkhead domain was highly conserved (see Fig. S1B in the supplementary material). Other than the forkhead domain, no other apparent domains were found, but the N-terminal and C-terminal regions showed somewhat similar sequences between LvfoxN2/3 and chordate foxN2 or foxN3 with 30% overall similarity.

Expression pattern of foxN2/3

The early expression pattern of L. variegatus foxN2/3 was similar to that previously reported for S. purpuratus (Tu et al., 2006), and with time the expression sites changed dynamically. Whole-mount expression of L. variegatus foxN2/3 was similar with time the expression sites changed dynamically. Whole-mount expression of L. variegatus foxN2/3 was similar.
Fig. 1. Dynamic expression pattern of FoxN2/3 in the endomesoderm. (A-J) Whole-mount in situ hybridization of foxN2/3. At the 16- and 128-cell stages, foxN2/3 is not expressed (A,B). At the hatched blastula stage, foxN2/3 is expressed in the vegetal plate (C,D). At the mesenchyme blastula stage, foxN2/3 is gone from the ingressed PMCs and is expressed in the remaining endoderm (E,F). By the end of mesenchyme blastula stage, foxN2/3 is expressed in endodermal cells (G,H). By late gastrula stage, foxN2/3 is expressed in the mid- and hindgut, with the foregut displaying weak foxN2/3 expression (I,J).

(K-R) Double fluorescence in situ hybridization of foxN2/3 with tissue specific markers. foxN2/3 expression is compared with the PMC marker tbr at the hatched blastula stage (K,L) and early mesenchyme blastula stage (M,N) and with the NSM marker gcm at the early mesenchyme blastula stage (O,P) and late mesenchyme blastula stage (Q,R). C, E, G, I, K, M, O and Q show the lateral view and D, F, H, I, L, N, P and R show the vegetal view. In both P and R FoxN2/3 is expressed in a full ring though the embryos shown focus on the GCM half embryo, making it appear, incorrectly, that FoxN2/3 is expressed asymmetrically. The full ring can be seen in F and H.

mesoderm (NSM) cells (Fig. 1E,F), which later form muscle, coelomic pouch, blastocoelar and pigment cells. Over time foxN2/3 expression was reduced to a low level in the NSM and appeared in endoderm by the late mesenchyme blastula stage (Fig. 1G,H) where it continued to be expressed during archenteron elongation (Fig. 1I,J). A comparison of foxN2/3 in L. variegatus and S. purpuratus (Tu et al., 2006) suggests a similar dynamic pattern of expression through development with the exception that in L. variegatus, foxN2/3 mRNA was expressed in the endoderm from the early gastrula stage and remained in the mid- and hindgut area through the late gastrula stage (which was not reported in S. purpuratus). Thus, the spatial pattern of foxN2/3 expression is dynamic and sequentially covers most of the endomesodermal domains over time.

To confirm the lineage assignments, double fluorescence in situ hybridization was performed with foxN2/3 and two lineage specific markers: tbr (a PMC marker) and gcm (an NSM marker). At the hatched blastula stage, foxN2/3 was expressed in the precursors of PMCs as the expression sites of foxN2/3 and tbr were coincident (Fig. 1K,L). At the early mesenchyme blastula stage, as the PMCs ingressed they continued to express tbr but eliminated foxN2/3 (Fig. 1M,N); at the same time, foxN2/3 expression was present in the mesoderm surrounding PMCs in the vegetal plate where its expression overlapped with gcm and was observed in some endodermal cells outside gcm territory (Fig. 1O,P). At the late mesenchyme blastula stage, transcripts of foxN2/3 were strongly detected in endodermal cells and greatly reduced in the mesodermal cells in the vegetal plate (Fig. 1Q,R).

Gene regulatory network regulation of foxN2/3 expression

We next turned to the question of how foxN2/3 is regulated. The endomesodermal GRN model of August 2010 (http://sugp.caltech.edu/endomes/) indicates only an input from β-catenin to drive foxN2/3 expression in the micromere lineage. Earlier work showed that β-catenin enters the nuclei of micromeres at the 16-cell stage and is necessary to activate the entire micromere GRN (Logan et al., 1999). This occurs five hours before foxN2/3 is expressed in the hatched blastula suggesting that TFs downstream of β-catenin later activate foxN2/3 expression, or are necessary along with β-catenin. The basis for β-catenin as the activator of FoxN2/3 comes from elimination of β-catenin by injection of excess cadherin cytoplasmic tail (Logan et al., 1999; Davidson et al., 2002). With that background in mind, we used the network model to initiate analysis of foxN2/3 activation and look for additional activators of foxN2/3.

Because foxN2/3 has a dynamic pattern of expression over time, we expected its regulation to be more complex than just an input from β-catenin. Previously, a set of transcription factors including β-catenin, otx, blimp1, fox11/13b and even-skipped were shown to have similar expanding torus patterns of expression (Logan et al., 1999; Smith et al., 2008; Smith et al., 2007). Smith et al. showed that a subcircuit composed of otx, wt1β and blimp1 work together to produce their characteristic expanding expression pattern (Smith et al., 2007).

As the foxN2/3 expression region expands behind the expanding blimp1 expression pattern (Fig. 2A-D), Blimp1 was a promising candidate to regulate the dynamic foxN2/3 expression pattern. In a test of this hypothesis, however, knockdown of Blimp1b did not inhibit foxN2/3 expression in the vegetal plate (Fig. 2E-H). As a control for this experiment to verify that the blimp1b morpholino blocked Blimp 1 translation, in situ hybridization analysis showed that blimp1 expression expanded in the presence of the Blimp 1 morpholino, a signature pattern expected of a transcription factor.
that represses itself (see Fig. S2A-F in the supplementary material) (Smith et al., 2007; Livin and Davidson, 2006). Further, as expected, the blimp1b morpholinio also altered the endomesoderm GRN state resulting in a failure of archenteron invagination (see Fig. S2G,H in the supplementary material). Despite the inhibition of Blimp1b, foxN2/3 continued to be expressed at the vegetal plate of the blimp1b-MASO-injected embryos at the same point in time at which it was expressed in the control embryos, and it later expanded to other endomesodermal cells as well. Thus, though foxN2/3 and the genes in the blimp1 subcircuit showed highly similar expression dynamics, we conclude that foxN2/3 expression in micromeres, mesoderm and endoderm does not depend on that subcircuit for its expanding pattern of expression, and therefore it is very unlikely that β-catenin alone is the sole input.

If the blimp1 expanding torus is not involved, how is foxN2/3 activated? Here, we focused on activation of FoxN2/3 in the large micromeres. Several candidate PMC-specific transcription factors were chosen based on their position in the micromere GRN. We perturbed each gene and performed WISH to assess the change of foxN2/3 expression at the vegetal plate area. First, we tested pmar1, one of the earliest transcription factors of PMC specification. According to previous reports (Oliveri et al., 2003; Revilla-i-Domingo et al., 2007), pmar1 overexpression activates the PMC GRN by repression of the universal repressor hesC and transforms almost every cell in the embryo into a PMC. When we overexpressed pmar1 by mRNA injection, foxN2/3 expression was indeed induced in most of the cells (Fig. 3B). In other studies, this same result was noted only for micromere GRN genes and not for genes in any of the other cell types (Oliveri et al., 2008). This implies that Pmar1 is a component of the upstream circuit leading to foxN2/3 expression in PMCs. However, pmar1 expression in micromere progeny disappears earlier than the induction of foxN2/3 and therefore is unlikely to be proximal to foxN2/3 expression. Thus, we moved on to several candidate transcription factors, each of which is known to be regulated by Pmar1 double repression and expressed earlier than foxN2/3 induction: alx1, ets1 and tbr.

alx1, a PMC-specific transcription factor, is expressed in cells of the large micromere lineage after the fifth cleavage and is maintained by double repression of Pmar1 and/or Ets1. It is involved in early specification of micromeres and regulation of skeletogenic genes (Ettenson et al., 2003). alx1 MASO injection inhibited ingestion and further skeletogenesis, as expected, but loss of Alx1 did not prevent foxN2/3 expression in the precursors of PMCs, indicating that Alx1 expression does not occur upstream of foxN2/3 activation (Fig. 3D).

ets1 is a second downstream target of Pmar1 double repression and is known to be involved in the expression of alx1 and tbr, as well as other skeletogenic genes. Previously, Rottinger et al. showed that Ets1 is activated by phosphorylation, and the inhibition of MAPK by U0126 prevents Ets1 activity as a transcription factor in the precursors of PMCs (Rottinger et al., 2004). As shown in Fig. 3F, U0126 treatment inhibits foxN2/3 expression in the precursors of PMCs. As an independent test of an Ets1 input, a dominant-negative construct of Ets1 (Kurokawa et al., 1999; Sharma and Ettenson, 2010) was tested. Expression of dominant-negative Ets1 blocked foxN2/3 expression in the micromeres (Fig. 3H). Later, at the mesenchyme blastula stage, foxN2/3 expression was observed in endomesodermal cells other than micromere/PMCs in embryos expressing dnEts1. This result is consistent with the conclusion that Ets1 is involved only in the expression of foxN2/3 in micromeres, and not in the other cell types (see Fig. S3B in the supplementary material).

tbr is another transcription factor downstream of Pmar1 and Ets1, and is involved in the early specification of micromeres. Embryos injected with tbr morpholinos failed to express foxN2/3 in micromeres (Fig. 3J). In the same experiment, tbr MASO-injected embryos showed normal expression of other markers compared with the controls (see Fig. S4 in the supplementary material). The specificity of the tbr MASO was confirmed by a rescue experiment in which the repression of foxN2/3 by the tbr MASO was rescued by co-injection of tbr mRNA (Fig. 3K). In the tbr MASO-mRNA co-injected embryos and in tbr overexpressing (OE) embryos (data not shown), foxN2/3 expression was limited to the vegetal plate and did not show ectopic expression. This implies that Tbr is not sufficient to induce expression of foxN2/3 on its own, and requires other PMC-specific transcription factor(s) in an AND logic function (Tbr AND another TF). Thus, in micromeres, Pmar1 probably regulates foxN2/3 expression through Ets1 and/or Tbr, but not Alx1. As Ets1 also regulates Tbr, we cannot at present distinguish whether Ets1 activates foxN2/3 directly, or works indirectly through its role in activation of tbr. Further, as β-catenin activates pmarn1, which activates ets1 and tbr through its double repression gate, one cannot conclude that β-catenin has a direct input into activation of foxN2/3.

The expression of foxN2/3 in PMCs, non-skeletogenic mesoderm and endodermal cells can be regulated by either one common induction/repression mechanism similar to the Wnt-Blimp-Otx torus subcircuit, or by different independent mechanisms. We first asked whether foxN2/3 has an upstream role in the dynamic expression of foxN2/3 in other cells. Two MASOs were designed and injected into fertilized eggs to inhibit the endogenous translation of foxN2/3 and both of the MASOs showed similar effects on early development, though the foxN2/3
MASO 1 optimal dosage was lower than that for foxN2/3 MASO 2. As controls, the efficiency and specificity of foxN2/3 MASOs was shown by repression of red fluorescent protein (RFP) using the morpholino complementary to the FoxN2/3 5’ UTR sequence. The expression of FoxN2/3 5’ UTR-connected membrane RFP was eliminated in the presence of the foxN2/3 morpholino whereas membrane RFP downstream of reverse strand FoxN2/3 5’ UTR was not repressed (see Fig. S5 in the supplementary material). In Fig. 5, another control shows that introduction of FoxN2/3 mRNA rescues the FoxN2/3 MASO. Thus, the morpholinos, by those tests, are efficient and specific for blockage of FoxN2/3 translation.

When foxN2/3 translation was blocked by MASO injection, foxN2/3 was still transcribed at the vegetal pole area at the hatched blastula stage (Fig. 4A-D). Importantly, its expression expanded to adjacent cells later and QPCR results also showed that the expression level of foxN2/3 was not significantly changed by foxN2/3 MASOs (ΔΔCt<2). These results indicate that FoxN2/3 does not regulate itself, nor does its expression in one set of cells participate in the expanding torus pattern of foxN2/3 expression, i.e. later expression does not depend on the earlier expression. As an independent test of this hypothesis, when we removed micromeres at the 16-cell stage, foxN2/3 was still expressed in the vegetal plate at roughly the same time that foxN2/3 was expressed in the non-skeletogenic mesoderm of control embryos (Fig. 4E-H). Thus, foxN2/3 expression in non-skeletogenic mesoderm cells is not dependent on FoxN2/3 expression in micromeres. Moreover, its expression is not dependent on signals from the micromeres. Because Delta is known to be important for NSM specification, perturbation of Delta signaling from micromeres was tested and the results suggest that FoxN2/3 is activated in NSM independent of Delta signaling. When the Delta signaling pathway was disrupted by dominant-negative notch mRNA or delta MASO injection, the embryos showed the albino phenotype as expected from previous reports using these reagents (Croce and McClay, 2010; Sherwood and McClay, 1999; Sweet et al., 2002). However, foxN2/3 was still expressed in the vegetal plate (Fig. 4I-L; data not shown). Thus, the later expression of foxN2/3 requires an independent mechanism that is not dependent on earlier foxN2/3 expression in micromeres, nor from the micromere-released signals, including activation of Delta in the NSM. foxN2/3 has at least one PMC-specific cis-regulatory module that is regulated by Ets1 and/or Tbr, and as Tbr is only expressed by micromeres, this module is not required for the later endomesodermal foxN2/3 expression. These results suggest that multiple cis-regulatory modules sequentially regulate activation of foxN2/3 to produce its dynamic pattern of expression in embryos.

**Functional characterization of FoxN2/3 in micromeres**

Next, we examined the functional role of FoxN2/3 in micromeres. foxN2/3 expression was perturbed in a number of experiments with the morpholinos. PMC ingestion was not observed in the foxN2/3 MASO-injected embryos by the time the control embryos had finished PMC ingestion (n=57/65; Fig. 5A,C). Archenteron invagination in foxN2/3 MASO-injected embryos was also delayed relative to controls and there was a reduced frequency of fusion to form a stomodeum. Without FoxN2/3, formation of larval skeleton was also disrupted; at 24 hours post fertilization, the control embryos had formed typical elongated spicules, whereas FoxN2/3 KD embryos did not yet have any skeletons (n=37/39; Fig. 5B,D). Eventually, spicules were observed in some foxN2/3 MASO-injected embryos, but the skeleton was disorganized and the length
of the spicules was shorter than that of the controls. As the MASO concentration was increased, the spicule defects became more severe. Thus, reduction of FoxN2/3 caused a delay or loss of skeletogenesis and endoderm development. In addition, overexpression of FoxN2/3 by mRNA injection caused precocious ingestion with little delay of archenteron development but showed no further defects (data not shown). Given this preliminary survey of visible phenotypes upon perturbations, we next turned to experimental analysis of function in a more targeted approach at a cellular and molecular level.

**FoxN2/3 is necessary for syncytium formation and for inhibition of transfating**

To focus on the PMC-specific function of FoxN2/3, experiments were designed so that foxN2/3 MASOs were exclusively carried by PMCs. For this cell-specific inquiry, micromere transplantation experiments combined micromeres from MASO-injected embryos with control micromereless (micromere-removed) embryos (Fig. 6A-F). As controls, four unperturbed micromeres labeled with the fluorescent dye FITC were transplanted to unlabeled micromereless embryos. The descendants of those control micromeres underwent normal skeletogenesis and cells with green fluorescence were located along the larval skeleton (Fig. 6A-C). By contrast, PMCs originating from foxN2/3 MASO-injected micromeres remained in the blastocoel without contributing to the larval skeleton (Fig. 6D-F). Surprisingly, whereas the FoxN2/3 KD micromeres failed to form skeleton, the recombinant embryos had a normal skeleton produced by cells without fluorescent dye $(n=3/3$ and $3/4$, two independent experiments; Fig. 6F). These data suggested that the skeletogenic cells had not originated from the transplanted micromeres, but instead had transfated from endomesodermal cells of the host embryo. In the absence of PMCs, other mesoderm cells are known to transfate to PMCs and form the skeleton (Ettensohn and McClay, 1988). Thus, the presence of these transfated cells suggests that in the absence of FoxN2/3 in micromeres, there is a failure to send signals that usually inhibit the transfating of non-skeletogenic mesoderm cells. Further, these results imply that FoxN2/3 is required for syncytium formation, as FoxN2/3 KD PMCs failed to participate in the transfated syncytium.

To challenge experimentally the question of syncytium formation even more directly, a mixed micromere experiment was performed. First, two red control and two green control micromeres were transplanted at the 16-cell stage into the space left behind by removal of micromeres from an unlabeled host (Fig. 6G). After the formation of syncytium, the red and green fluorescent dyes mixed in the syncytium and resulted in yellow cells along the larval skeleton (Fig. 6H, I), indicating that the control red and control green cells had collaborated in syncytium formation. By contrast, when two red micromeres injected with foxN2/3 MASO were transplanted along with two control green micromeres, the green PMCs contributed to the skeleton, but the FoxN2/3 KD PMCs remained in the blastocoel separate from the skeleton $(n=3/3$ and $1/2$, two independent experiments; Fig. 6J, K). Thus, knockdown of FoxN2/3 in PMCs resulted in a failure of these cells to be incorporated into the syncytium, explaining at least one of the reasons that the larval skeleton is malformed in FoxN2/3 KD embryos.

As a further independent test, as the function of FoxN2/3 was shown to be downstream of Tbr, we predicted that knockdown of Tbr should also produce some, if not all, of the defects shown by knockdown of FoxN2/3. Accordingly, the same micromere transplant experiments were performed with the tbr morphants (Fig. 6L, M). As would be predicted if tbr is a major regulator of foxN2/3, tbr morphant PMCs also failed to produce a larval skeleton and failed to prevent non-skeletogenic mesoderm transfating; the recombinant embryos had a normal skeleton produced by cells without fluorescent dye $(n=3/3$ and $3/3$, two independent experiments; Fig. 6M).
Fig. 7. foxN2/3 and PMC-specific gene expression. (A-D) 1d5 immunostaining labeling Msp130. Msp130 is expressed in the control embryo in the PMCs at the early gastrula stage (A,B). Msp130 expression is greatly delayed in foxN2/3 MASO-injected embryos (C,D). (E-J) Whole-mount in situ hybridization of VEGFR. VEGFR is expressed in the ingressed PMCs of control embryos (E-G). VEGFR is not expressed in foxN2/3 KD embryos (H,I), but appears later (J). EG, early gastrula; LG, late gastrula; MB, mesenchyme blastula; Pr, prism.

**FoxN2/3 is necessary for skeletogenic gene expression**

To understand further the molecular function of foxN2/3 in PMCs, we looked at the expression of potential downstream target genes of FoxN2/3 in the PMC GRN. First, Msp130, a PMC-specific cell-surface glycoprotein involved in skeletogenesis, was examined by immunostaining (Fig. 7A-D). foxN2/3-MASO injected embryos showed no expression of Msp130 ~12 hours after fertilization, when control embryos showed a high level of Msp130 in PMCs (Fig. 7A,C). Eventually, Msp130 expression was recovered in ingressed cells of foxN2/3 KD embryos (Fig. 7D, 20 hours after fertilization); whether these Msp130-expressing cells were the original PMCs or transfated PMCs was not determined, but the expression coincided with the appearance of the transfated PMCs in micromere-removed embryos (Ettensohn and McClay, 1988). When we examined other skeletogenic genes by QPCR in FoxN2/3 KD embryos, sm30 and sm50 expression levels were also strongly reduced, consistent with the hypothesis that many skeletogenic genes require FoxN2/3 input (Table 1). Eventually, expression of the skeletogenic genes assayed, i.e. MSP130, sm30 and sm50, recovered in later stages of FoxN2/3 KD embryos. Again, that ‘recovery’ might instead have been expression by transfated PMCs. In either case, these results indicate that FoxN2/3 is necessary for the early expression of skeletogenic genes. Another FoxN2/3 target was the VEGF Receptor (VEGFR), which is known to be involved in the proper organization of larval skeleton (Duloquin et al., 2007). WMISH showed that the expression of VEGFR in the PMCs is absent in FoxN2/3 KD embryos whereas the control embryos showed VEGFR signal in the ingressed PMCs (Fig. 7E-I). In FoxN2/3 KD embryos, VEGFR expression recovered during later gastrulation, just as other skeletogenic genes recover (Fig. 7J).

**Table 1. Effect of foxN2/3 morpholino antisense oligonucleotides (MASO) MO1 and MO2 on expression of the skeletogenic genes sm30 and sm50**

<table>
<thead>
<tr>
<th>FoxN2/3 MO1 (ΔΔCt)</th>
<th>FoxN2/3 MO2 (ΔΔCt)</th>
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<tbody>
<tr>
<td>sm30</td>
<td>-6.20±7.02</td>
</tr>
<tr>
<td>sm50</td>
<td>-4.42±8.52</td>
</tr>
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Embryos were harvested at the mesenchyme blastula stage. Ct (crossing point threshold) values determined by quantitative polymerase chain reaction were normalized by ubiquitin and ΔΔCt values were calculated by subtracting the sample from the control. Two separate sets of experiments are shown.

**DISCUSSION**

**Control of foxN2/3 expression in micromeres**

The dynamic expression pattern of FoxN2/3 is similar to the subcircuit of Blimp1, Wnt8 and Otx, as well as to their downstream genes including fox11/13b and eve (Smith et al., 2008; Smith et al., 2007). However, knockdown of Blimp1b failed to inhibit the foxN2/3 expression pattern. Instead, in micromeres, foxN2/3 expression requires Ets1 and Tbr, downstream of Pmar1 and β-catenin, for its activation. We cannot rule out a direct input from either the Pmar1 double repression system or β-catenin, though by the time FoxN2/3 is activated expression of both factors is reduced or absent in micromeres. Both, however, are upstream of expression of Ets1 and Tbr, the two TFs shown to be required and expressed at the time of FoxN2/3 activation. Inhibition of Ets1 activation or knockdown of Tbr did not inhibit foxN2/3 expression in non-skeletogenic mesoderm or endoderm cells, indicating that control of expression of foxN2/3 in skeletogenic mesoderm is independent of regulatory mechanisms controlling foxN2/3 elsewhere in the embryo. We were surprised to learn that in the mesoderm, foxN2/3 is activated independent of the Delta signal that is necessary for several core transcription factors of the NSM network subcircuit (Sherwood and McClay, 1999; Sweet et al., 2002). The endodermal expression of foxN2/3 beginning at mesenchyme blastula diverges into weak expression in the foregut and stronger expression in the mid/hindgut area during late gastrulation; this pattern implies that yet other regulatory modules exist in the foxN2/3 enhancer distinct from the mesodermal control module. Thus, our data suggest that at least three different cis-regulatory modules are employed for the expression of foxN2/3 during early development up to gastrulation.

This study on foxN2/3 expression highlights the benefits of using a GRN model as a guide to elucidate regulatory interactions. The PMC GRN provided a logical framework, allowing us to focus our efforts on candidates for the regulators of foxN2/3 expression in PMCs. Pmar1 is one of the earliest PMC-specific transcription factors activated by β-catenin nuclear localization and it regulates most downstream PMC-specific genes. Thus, it was expected that Pmar1 would be necessary for foxN2/3 expression in PMCs also, and our results support that hypothesis. The PMC GRN also provided candidates to explain the temporal difference between the modeled Pmar1 input, and the actual time of foxN2/3 activation. We predicted that foxN2/3 expression requires additional inputs from other components of the micromere GRN. The prior information quickly led us to Ets1 and Tbr, which were found to regulate foxN2/3 expression in PMCs. Thus, the PMC GRN was utilized as a map to find the proper niche of the gene in the PMC specification cascade. By extension, any gene can be examined in this way to find its position in the network, using the prior knowledge contained in GRN models.
Function of FoxN2/3 in micromeres and PMCs

Immunostaining, QPCR and WMISH data show that FoxN2/3 KD prevents the early expression of skeletogenic genes and other PMC-specific genes as part of what has been described as a parallel input of a number of transcription factors (Peter and Davidson, 2009). Thus, FoxN2/3 is indispensable for the circuity controlling PMC differentiation genes. This circuity takes the appearance of many parallel inputs because the GRN is responsible for activation of all PMC differentiation genes. Some of the PMC differentiation genes are known to receive direct inputs from a subset of those transcription factors, but unless a detailed cis-regulatory analysis is done it is not possible to distinguish between a requirement for the TF as part of a competent GRN state and a direct requirement for the TF in activation of specific downstream genes. Expression of foxN2/3 is extinguished in PMCs shortly after ingestion. These data suggest that the importance of FoxN2/3 in the micromeres is in achieving a competent GRN state rather than directly contributing to activation of the downstream genes. Of course this does not rule out possible direct inputs from FoxN2/3 into the differentiation genes, but if FoxN2/3 provides direct input, it must transfer continuing expression of those genes to other transcription factors shortly thereafter because it disappears from PMCs.

As shown previously, knockdown of the VEGF receptor (VEGFR) results in failure to produce a skeleton in embryos (Duloquin et al., 2007). Here, the data show that knockdown of FoxN2/3 in micromeres causes a large reduction in VEGFR expression by PMCs, yet eventually the embryos produce a skeleton. This was puzzling until we learned that transfated mesoderm cells produced the skeletons in FoxN2/3 KD embryos. However, in those experiments the transfated PMCs made a skeleton although the FoxN2/3 MASO was present in those cells also, implying that as a result of transfating, the neo-PMCs somehow got around the FoxN2/3-knockdown block to synthesize the VEGFR. This result implies both that FoxN2/3 is an indirect input into expression of the VEGFR, and that the transfating mechanism employs a network that no longer requires FoxN2/3. Indeed, it was shown earlier that transfated PMCs also do not require Pmar1 (Ettensohn et al., 2007). As we show that first cause differentiation, and then maintain it, remain to be solved in detail.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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