Development, Growth & Differentiation
The Japanese Society of Developmental Biologists

Develop. Growth Differ. (2014) **56**, 310–322 doi: 10.1111/dgd.12133

Original Article

Transcription factor Tbx6 plays a central role in fate determination between mesenchyme and muscle in embryos of the ascidian, *Halocynthia roretzi*

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Developmental fates are determined in relation to other fates in surrounding cells within the embryo, creating diversified tissue or cell types in the course of development. In ascidian embryos, maternally localized factors and inductive signals play essential and cooperative roles in fate determination at appropriate spatial and temporal positions during embryogenesis. Here, to clarify fate determination mechanisms of mesenchyme and muscle during mesenchyme induction by the fibroblast growth factor (FGF) signal in *Halocynthia roretzi*, we examined the function of transcription factor Tbx6, which acts as a pivotal mediator of the maternally localized muscle differentiation determinant Macho-1. Our results suggest that the level of *Tbx6* expression increases in muscle lineage cells through positive feedback regulation that promotes muscle differentiation as well as mesenchymal fate suppression. In addition, the FGF signal inactivated Tbx6 transcriptional activity and positive feedback, leading to induction of the mesenchymal lineage. Taken together, our finding suggests that Tbx6 is an important factor for determining mesenchyme and muscle fates.

Key words: ascidian, induction, mesenchyme, muscle, Tbx6.

Introduction

The mechanisms by which developmental fates are determined during embryogenesis have been extensively studied. Fate determination processes during cell cleavage stages are of particular interest because cell division often produces two daughter cells with distinct fates. Two well-known mechanisms for this phenomenon are determinant segregation and induction. The former process involves the segregation of a determinant factor only into one daughter cell during cell division, whereas the latter involves an extrinsic signaling molecule that directs the cell fate of only one daughter cell. In induction, the two daughter cells typically have equal or few intrinsic molecular differences. While the importance of these processes is well appreciated, it remains to be fully understood how the specific factors mediate cell fate determination.

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Received 5 December 2013; revised 9 March 2014; accepted 12 March 2014.

The ascidian embryo, *Halocynthia roretzi*, is particularly suited as a model organism for studying fate determination mechanisms because most developmental fates are determined by the 110-cell stage. This characteristic allows the individual mechanisms that comprise fate determination within the whole embryo to be studied with single-cell resolution (Kumano & Nishida 2007; Lemaire 2009). In addition, a wealth of experimental data have been accumulated for this species using both molecular and cellular techniques such as embryonic manipulation, including blastomere isolation and recombination, which are possible due to the large size of embryos. Due to these advantageous properties, fate determination mechanisms can be verified in *H. roretzi* embryos through multiple approaches.

In the posterior region of the vegetal hemisphere of ascidian embryos, mesenchymal fate is induced at the 32-cell stage by the fibroblast growth factor (FGF) signal transduction (Kim *et al.* 2000, 2007; Kim & Nishida 2001; Imai *et al.* 2002, 2003; Miya & Nishida 2003). The ascidian protein FGF9/16/20 serves as the inducer of the FGF signal (Imai *et al.* 2002; Kumano *et al.* 2006; Kim *et al.* 2007). The *FGF9/16/20* gene is expressed at the 32-cell stage in A6.3 and B6.1

blastomeres (Imai *et al.* 2002; Kumano *et al.* 2006; Fig. 1B,G), which function as inducer blastomeres during mesenchyme induction (Kim & Nishida 1999), and also in A6.1, A6.2, and A6.4 blastomeres (Imai *et al.* 2002; Kumano *et al.* 2006; Fig. 1B,G), which serve as inducers for the induction of other tissues such as the notochord and brain, in the anterior embryonic region (Nishida & Satoh 1989; Nakatani & Nishida 1994; Miyazaki *et al.* 2007).

The induction of mesenchyme and other tissues requires not only the extrinsic inductive FGF signal, but also an intrinsic competence factor that responds to the same FGF signal, allowing for different outcomes depending on the nature of the intrinsic factor (Kim *et al.* 2000; Kobayashi *et al.* 2003; Kumano *et al.* 2006; Kumano & Nishida 2009). One such competence factor involved in mesenchyme induction, Macho-1, is a maternally localized zinc-finger type transcription factor (Kobayashi *et al.* 2003) that also plays a deterministic role in muscle differentiation (Nishida & Sawada 2001; Sawada *et al.* 2005; Satou *et al.* 2002; Yagi *et al.* 2004).

Mesenchymal and muscle lineages are not differentiated until the 32-cell stage (Fig. 1B,F) and are only first separated by cell division at the 44- and 64-cell stages in the anterior (B6.2) and posterior (B6.4) mesenchymal and muscle lineages, respectively (green for mesenchyme and red for muscle in Fig. 1). Therefore, although mesenchyme and muscle precursor cells are likely intrinsically very similar, as both express Macho-1, they are distinguished by the FGF signal. This property has been confirmed experimentally by cell transplantation experiments in which changing the direction from which the FGF signal was applied by 180 degrees reversed the positioning of the mesenchymal and muscle tissues within the embryo (Kim

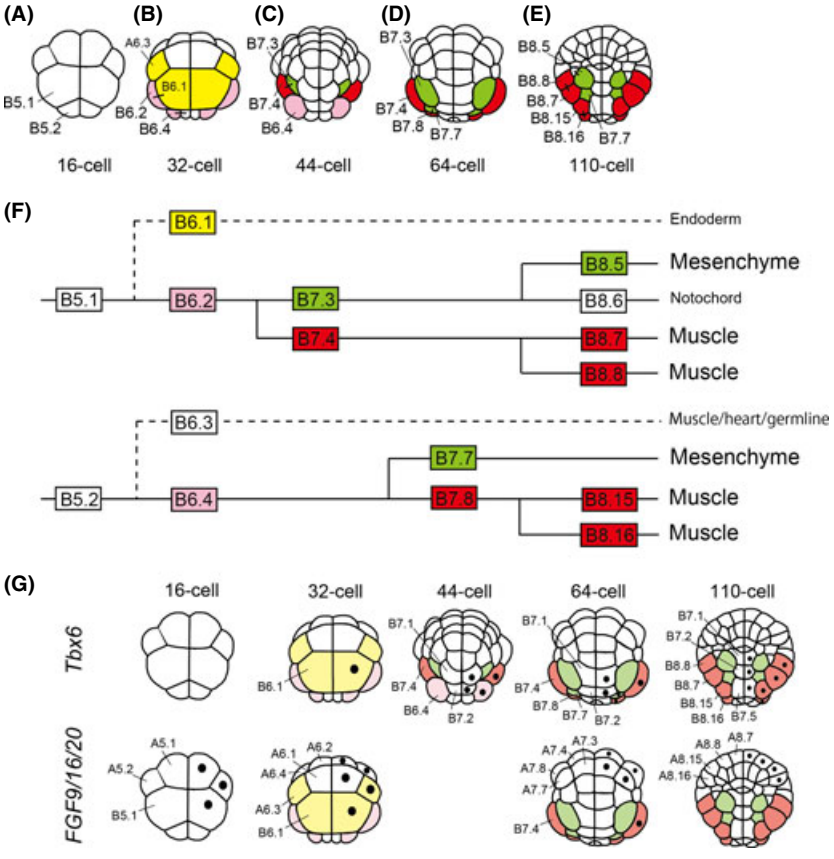

Fig. 1. (A–E) Schematic diagrams illustrating ascidian embryos. All embryos are shown in vegetal views with the anterior at the top. 16-cell (A), 32-cell (B), 44-cell (C), 64-cell (D) and 110-cell (E) stage embryos. The pink blastomeres are fated to become either mesenchyme- or muscle-lineage blastomeres, which are colored green and red, respectively. Yellow cells are endoderm and have been identified as sources of inductive signals for mesenchyme induction. (F) Lineage tree that starts from the posterior-vegetal blastomeres (B5.1 and B5.2) of the 16-cell stage embryo (A). Colors in the tree correspond to those in the embryos above (A–E). The branching positions of the tree reflect the timing of cell divisions as shown in the embryos above (A–E). (G) Schematic diagrams illustrating the expression patterns of *Tbx6* and *FGF9/16/20* (black dots in the cells). For easier visualization of the patterns, the colors of the respective blastomeres are shown in a lighter shade.

et al. 2007). Based on these findings, mesenchyme and muscle are considered to be induced and default developmental fates, respectively, with the precursor cells (B6.2 and B6.4) being directed towards either mesenchymal or muscle fates after subsequent cell division. Consistent with this proposed sequence of events, inhibition of the FGF signal by either the isolation of presumptive mesenchymal cells before induction, treatment with an inhibitor of MEK, which is a cytoplasmic component of the FGF signaling cascade, or morpholino oligonucleotide (MO)-mediated knockdown of *FGF9/16/20*, prevents mesenchymal differentiation and leads to ectopic muscle formation in the original mesenchyme lineage (Kim & Nishida 1999, 2001; Imai *et al.* 2002, 2003; Kim *et al.* 2007).

Following segregation of the mesenchymal and muscle lineages in the ascidian species *Ciona savignyi* and *C. intestinalis*, the bHLH domain transcription factor Twist-like 1 is activated and regulates the expression of a number of downstream mesenchyme-specific genes (Imai *et al.* 2003; Tokuoka *et al.* 2004, 2005). *Twist-like 1* is first expressed in the mesenchyme lineage at the 64-cell stage right after the two-lineage segregation (Imai *et al.* 2003). The expression of *Twist-like 1* is under control of the FGF signal (Imai *et al.* 2003; Tokuoka *et al.* 2004). Another gene *Twist-like 2* is also expressed specifically in the mesenchyme cells and is located downstream of Twist-like 1 (Imai *et al.* 2003). In the muscle lineage, Macho-1 activates transcription of the gene encoding a T-box transcription factor Tbx6, which regulates the expression of several downstream muscle-specific genes, in both *Halocynthia* and *Ciona* embryos (Mitani *et al.* 1999, 2001; Yagi *et al.* 2004, 2005; Sawada *et al.* 2005; Fig. S1). Accordingly, Twist-like 1 and Tbx6 are considered to be key transcription factors for mesenchyme and muscle fate determination, respectively (Nishida 2005).

In the present study, we first attempted to clarify the temporal and spatial profiles of mesenchyme induction and muscle fate suppression, and identified a previously unknown source of the FGF signal for muscle suppression. In addition, we also found a possible molecular mechanism by which the FGF signal suppresses muscle differentiation in a transcription-independent manner. Based on our findings, we proposed a two-step model for mesenchyme induction.

Materials and methods

Animals and embryos

Adults of *H. roretzi* were collected in the vicinity of the Asamushi Research Center for Marine Biology and the Otsuchi International Coastal Research Center, and kept in tanks during the spawning season. Eggs were spawned under temperature- and light-controlled conditions, fertilized with a suspension of non-self sperm, and allowed to develop in Millipore-filtered seawater containing 50 μg/mL of streptomycin and kanamycin at 11°C.

Isolation of Twist-like by 5′RACE and 3′RACE

Homology searches against the *Halocynthia* expression sequence tag (EST) database MAGEST (Kawashima *et al.* 2000, http://magest.hgc.jp/) with *Ciona Twist-like* gene sequences identified a clone (NR007C24) as a candidate *Halocynthia* orthologue of the *Twist-like* gene. 5′RACE and 3′RACE were performed with the SMART RACE cDNA Amplification Kit (Ambion) and the 643-bp sequence of NR007C24, to obtain a full-length cDNA sequence. One 5′RACE and two 3′RACE fragments were obtained that completely matched the sequence of NR007C24. One 3′RACE fragment had a 242-bp deletion from the other, resulting in a different position of the stop codon. Therefore, two *Twist-like* cDNAs were present, consisting of 1830 and 1588 nucleotides and encoding predicted proteins of 164 and 171 amino acids, respectively.

Morpholino oligonucleotides and RNA injection

Specific morpholino antisense oligonucleotides (MO, Gene Tools) to knockdown the expression of *FGF9/16/20* and *Macho-1* were prepared as described previously (Kobayashi *et al.* 2003; Kumano *et al.* 2006). The sequence of the MO to knockdown the expression of *Tbx6* was 5′-CGGAGATAGGAAAAGCTGACATAGC-3′ (translation start site is underlined), and the specificity of the MO was confirmed by its ability to abrogate *Muscle actin* expression after injection into embryos. In addition, the injection of *Tbx6* RNA (see below) was able to rescue the MO-induced knockdown of *Muscle actin* expression (Fig. S1). The standard control morpholino oligonucleotide provided by Gene Tools was used as a control.

Plasmids for *in vitro Tbx6* RNA synthesis were prepared as follows. Polymerase chain reaction (PCR)-amplified fragments containing the entire open reading frame (ORF) of *Tbx6*, partial 5′ and 3′ UTRs with or without a modified sequence 5′ to the translation start site (ATGTCAGCTTTTCCTATCTCCGAT replaced with ATGAGCGCATTCCCAATTAGCGAT) were sub-cloned into plasmid pBS-HTB (N). The modified sequence encoded for the original amino acid sequence of Tbx6, but the corresponding RNA would no longer serve as a target for the *Tbx6* MO. Plasmids for *in vitro* RNA

synthesis of the C-terminal (PPTP>PPAP) and the T-box domain (PNSP>PNAP) mutants of *Tbx6* RNA were constructed as follows. First, target fragments were amplified by inverse PCR using the plasmid containing the unmodified *Tbx6* gene sequence as a template and KOD polymerase (Toyobo) with the primer sets 5′-CGTCGGACGATGACGCTAGCAATCT-3′ and 5′-GTGCAGGTGGTGATGATCCAATGCC-3′ (substitution underlined) for the C-terminal mutant, and 5′-AG CCAACGGTTCGAAGTGGATGA-3′ and 5′-GGCGCA TTCGGATGCAAGAAAAATC-3′ (substitution underlined) for the T-box domain mutant. The amplified fragments were then digested with *Dpn* I and self-ligated after end-kinase treatment. Capped and poly(A)-tailed RNAs for *Tbx6*, *Tbx6* for rescue, and the C-terminal and T-box domain mutants were synthesized as described previously (Kumano *et al.* 2006) using the above plasmids as templates. Microinjection of the synthesized RNA and MO was carried out 45 min to 2 h after fertilization, as described previously (Miya *et al.* 1997). The injected amount was approximately one hundredth of the egg volume. The data presented in this study are the average of at least two independent injections.

MEK inhibitor treatment and cell cleavage arrest

Cell cleavage was inhibited according to the method described by Kobayashi *et al.* (2003). Treatment with the MEK inhibitor (MEKi) UO126 (Promega) at 2 μmol/L was performed as described previously (Minokawa *et al.* 2001).

In situ *hybridization and immunohistochemistry*

Detection of mRNA for *Muscle actin* (Kusakabe *et al.* 1991), *Tbx6* (Yasuo *et al.* 1996) and *Twist-like* by whole-mount *in situ* hybridization was performed as described previously (Wada *et al.* 1995; Kumano *et al.* 2011). Immunohistochemical staining for mesenchyme-specific Mch-3 antigens (Kim & Nishida 1998) was carried out as described previously (Kobayashi *et al.* 2003). Fluorescent images were acquired with a BX61 fluorescence microscope (Olympus).

Results

Spatial analysis of muscle fate suppression

We first performed MEKi treatment at various times during the cleavage stages and examined whether muscle fate was suppressed in the mesenchyme lineage by whole mount *in situ* hybridization for *Muscle actin*.

Embryos treated with MEKi from the 32-cell stage showed ectopic *Muscle actin* expression in nearly all cells of both the anterior (B8.5, 97.2% [n = 36]) and posterior (B7.7, 100% [n = 36]) mesenchyme lineages at the 110-cell stage (Fig. 2A,B). Treatment starting from the 44-cell stage resulted in ectopic expression in all posterior-lineage blastomeres (100% [n = 38]), but in only approximately 60% of those of the anterior lineage (60.5% [n = 38]) (Fig. 2C,C',C"). This trend of reduced *Muscle actin* expression in the anterior lineage continued when MEKi treatment was initiated at the 64-cell stage, as no anterior- (0% [n = 40]) and 57.5% (n = 40) of posterior-lineage blastomeres exhibited ectopic expression (Fig. 2D). These results are consistent with the observed expression rates of the late mesenchymal differentiation marker Mch-3 (Kim & Nishida 1998) in embryos treated with MEKi from the 64-cell stage: 85.4% (n = 48) of anterior and only 29.2% of posterior mesenchyme-lineage cells showed its expression (data not shown). Together, these results suggest that the FGF signal is necessary during the cleavage stages to suppress muscle differentiation as well as to induce the mesenchyme fate. They also demonstrate that FGF-mediate suppression and induction complete earlier in the anterior than the posterior lineages.

Finally, almost no cells in the mesenchyme lineage exhibited *Muscle actin* expression when the MEK signal was blocked from the 76-cell stage 0% (n = 40) in B8.5 and 7.5% (n = 40) in B7.7 (Fig. 2E). This result contrasts with the previous result that mesenchyme induction was still not complete at the 110-cell stage as only 44% of embryos treated with MEKi from this stage expressed Mch-3 (Kim & Nishida 2001). Therefore, these results suggest that induction of the mesenchyme fate (Mch-3 expression) requires continuous MEK activity even after muscle fate is suppressed (no *Muscle actin* expression).

Temporal analysis of muscle fate suppression

Previous blastomere recombination experiments identified A6.3 and B6.1 as sources of mesenchyme induction in the anterior B8.5 lineage, and B6.1 as a source for the posterior B7.7 lineage (Kim & Nishida 1999). We next locally inhibited the FGF signal by injecting individual blastomeres of cleaving embryos with an MO against *FGF9/16/20* and examined *Muscle actin* expression. The result showed that the FGF signal from not only B6.1, but also B6.2, is required for suppression of the muscle fate in B7.7 (Fig. 2F,G, H). In the injection targeting B6.1 or B6.2 blastomeres, ectopic *Muscle actin* expression was not observed in the anterior B8.5 lineage, suggesting that

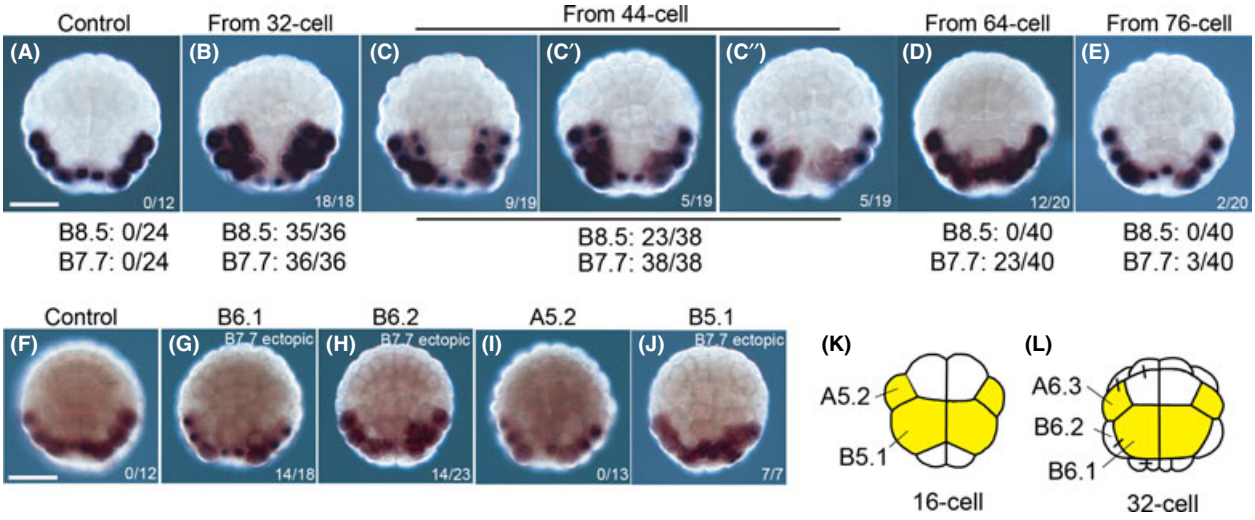

Fig. 2. (A–E) Expression of *Muscle actin* visualized by *in situ* hybridization in 110-cell stage embryos treated with MEK inhibitor or DMSO (dimethylsulfoxide) (control) from the indicated stages (A). The numbers in the lower right-hand corners of the images indicate the number of embryos with ectopic expression in at least in one mesenchyme lineage cell. In the case of MEK inhibitor treatment from the 44-cell stage, representative images and the numbers of embryos with ectopic expression in all mesenchyme cells (C), in cells on both sides (left and right) of the embryo (C'), or in cells on either side (C'') are shown. The numbers below the images indicate the number of B8.5 or B7.7 cells with ectopic expression. (F–J) Expression of *Muscle actin* visualized by *in situ* hybridization in 110-cell stage embryos in which the blastomeres indicated above the images were injected with 3 μg/μL morpholino oligonucleotide (MO) against *FGF9/16/20* (G–J). The non-injected control embryo is also shown in (F). Injection of control MO did not affect *Muscle actin* expression (Fig. S1B). The numbers in the lower right-hand corners of the images indicate embryos showing ectopic expression in B7.7 blastomeres. Only one embryo with ectopic expression in B8.5 was obtained in this experiment and resulted when B5.1 was injected with the MO against *FGF9/16/20* (data not shown). Scale bars in (A) and (F): 100 μm. (K,L) Schematic diagrams of 16-cell (K) and 32-cell (L) stage embryos with the lineage of the sources of inductive signals for mesenchyme induction colored in yellow.

neither B6.1 nor B6.2 are required for anterior muscle fate suppression. It is possible that the MO injection at that stage had effect only in the posterior lineage as this lineage requires later exposure to the FGF signal for muscle fate suppression. However, this scenario is not likely because MO injection into B5.1, the mother cell of B6.1 and B6.2 at one-cell cycle earlier, still resulted in 14.3% (n = 7) of B8.5 blastomeres showing ectopic *Muscle actin* expression, while it resulted in 100% (n = 7) of B7.7 (Fig. 2J). Injection into A5.2, the mother cell of A6.3, of 16-cell stage embryos also did not result in ectopic *Muscle actin* expression in the anterior lineage (Fig. 2I), suggesting that the FGF signal from A6.3 (an A5.2 daughter) is not required for muscle fate suppression, although it is still possible that the injection was too late. Taken together with the previous finding that simultaneous FGF knockdown in the A- and B-lineages by injecting A4.1 and B4.1 blastomeres at the 8-cell stage resulted in ectopic *Muscle actin* expression in the anterior mesenchyme lineage (Kim *et al.* 2007), B6.1 and A6.3 blastomeres may play a redundant role, with only one of these being sufficient for anterior muscle fate suppression.

Tbx6 suppresses mesenchyme fate

To further elucidate how muscle and mesenchyme fates are determined, we first attempted to isolate an early marker for mesenchymal differentiation to be used in conjunction with Mch-3. Here, we identified a *Halocynthia* orthologue of *Twist-like* by a combination of EST database searches and RACE analysis (Fig. 3A). The expression of *Twist-like* in *Halocynthia*, as revealed by *in situ* hybridization, was first detected in the B7.7 mesenchyme lineage at the mid-gastrula stage (Fig. 3B,C, black arrowheads), which is markedly later than the timing of mesenchyme fate determination (late 64-cell stage, Kim & Nishida 1999) and the onset of *Twist-like 1* expression in *Ciona savignyi* (64-cell stage; Imai *et al.* 2003). The *Halocynthia* orthologue of *Twist-like* was expressed specifically in the mesenchyme lineage (Fig. 3C–E) until the tailbud stage, during which the gene was expressed at low levels in trunk lateral cells (white arrowhead in Fig. 3F). Thus, the identified Twist-like orthologue was used in subsequent experiments as an early marker for mesenchyme differentiation. We surveyed a *Halocynthia roretzi* genome database using this Twist-like

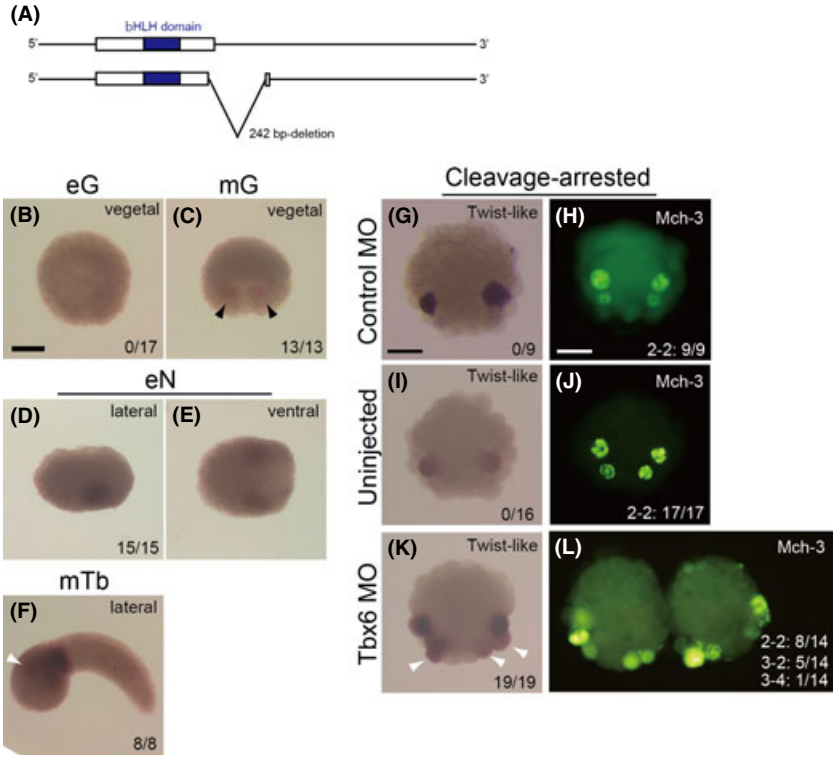

Fig. 3. (A) Schematic diagrams of two *Twist-like* cDNAs isolated in this study. One cDNA (bottom) contained a 242-bp deletion compared to the other (top), generating different amino acid sequences in the C-terminal regions. Open reading frames are indicated by open boxes and included bHLH domains (blue boxes). The nucleotide sequences of *Twist-like* have been deposited in the DNA Data Bank of Japan (DDBJ)/GenBank/European Molecular Biology Laboratory (EMBL) databases under the accession Nos. KJ427805 and KJ427806. (B–F) Expression of *Twist-like* revealed by *in situ* hybridization. Vegetal views of an early (B) and mid (C) gastrulae, and lateral and ventral views of early neurulae (D,E) and a mid tailbud (F). Anterior is to the top in (B) and (C), and to the left in (D), (E) and (F). The numbers in the lower right-hand corners of the images indicate embryos showing *Twist-like* expression. *Twist-like* expression was first detected in mesenchyme cells at the mid-gastrula stage (black arrowheads in C), and persisted to the tailbud stage, during which slight expression was also observed in trunk lateral cells (white arrowheads in F). (G,I,K) Expression of *Twist-like* in cleavage-arrested embryos that were not injected (I), or injected with 3 μg/μL control morpholino oligonucleotide (MO) (G) or 3 μg/μL *Tbx6* MO (K). *Twist-like* expression was strongly detected at the equivalent of the mid-tailbud stage in anterior mesenchyme lineage cells, but only weakly in posterior mesenchyme lineage cells of the control embryos at this stage (G,I). The numbers in the lower right-hand corners of the images indicate embryos with ectopic expression (white arrowheads in K). (H,J,L) Expression of the late mesenchymal marker Mch-3 in cleavage-arrested embryos that were not injected (J), or injected with 3 μg/μL control MO (H) or 3 μg/μL *Tbx6* MO (L). The numbers in the lower right-hand corners of the images indicate embryos with positive cells (e.g. 2–2 means two positive cells on each side of the embryo). Vegetal views with anterior to the top (G–L). Scale bars in (B), (G) and (H): 100 μm.

orthologue as query and found two other genes that showed sequence similarity (P. Lemaire, H. Sawada and H. Nishida, pers. comm., 2014).

We next investigated whether the muscle transcription factor, Tbx6, suppresses mesenchyme differentiation in early developmental stages. Knockdown of *Tbx6* by injecting eggs with a specific MO against *Tbx6* (Fig. S1) resulted in ectopic expression of *Twist-like* and Mch-3 in cells located at the peripheral posterior region of embryos (Fig. 3G–L). Based on their location, these cells were most likely muscle-lineage cells. These results suggest that Tbx6 plays a role in suppressing *Twist-like* expression and mesenchyme fate, and that a mutual inhibitory mechanism exists for muscle and mesenchyme differentiation pathways, as is often observed in many fate determination events, particularly when the initial difference is subtle (see below for *Tbx6* expression).

FGF signal inhibits a positive feedback loop of Tbx6 *expression*

Suppression of muscle fate by the FGF signal may involve inhibition of *Tbx6* expression and function. *In situ* hybridization analysis for *Tbx6* revealed that it was mainly expressed in muscle- and endoderm-lineage

cells during the cleavage stages (Fig. 4A–I), a result that is consistent with a previous report (Yasuo *et al.* 1996; Fig. 1G), although we also found that *Tbx6* was expressed in B6.2 and B6.4 blastomeres at the 32-cell stage (Fig. 4), but was not expressed in B7.1 and B7.2 blastomeres at the 110-cell stage. This difference may have been due to the use of a different *in situ* hybridization protocol. Importantly, although B7.3 (anterior mesenchyme cell) did not express *Tbx6*, its expression was detected in the B6.2 mother cell (muscle- and mesenchyme-fate) and also in B7.7 (posterior mesenchyme cell) at the 64-cell stage, but not the 110-cell stage. These findings indicate that both mesenchyme and muscle lineage cells possess *Tbx6* mRNA immediately after their separation, but that the mRNA transcripts are later confined to the muscle lineage. MO-mediated knockdown experiments of *Macho-1* demonstrated that *Tbx6* expression was inhibited from the 32- to 110-cell stages (Fig. 4J–L), whereas MO-mediated knockdown of *Tbx6* only inhibited *Tbx6* expression at the 64- and 110-cell stages. (Fig. 4M–P). Together, these results suggest that expression of *Tbx6* is activated at the 32-cell stage by Macho-1 and subsequently maintained by *Tbx6* itself, forming a positive feedback loop, a speculation that is consistent with the presence of a T-box-binding autoregulatory motif in the 5′ flanking region of the *Tbx6* gene in *Halocynthia* (Takahashi *et al.* 2005). Although Macho-1 may also directly participate in the maintenance event, due to the lack of *Tbx6* expression at the 32-cell stage by *Macho-1* knockdown (Fig. 4J), it was impossible to determine whether the absence of Macho-1 would have also eliminated *Tbx6* expression at the 64- and 110-cell stages (Fig. 4K,L). In addition, MEKi treatment did not alter the *Tbx6* expression pattern at the 32-cell stage (Fig. 4Q,R; compare to 4A,B,F,G), but did result

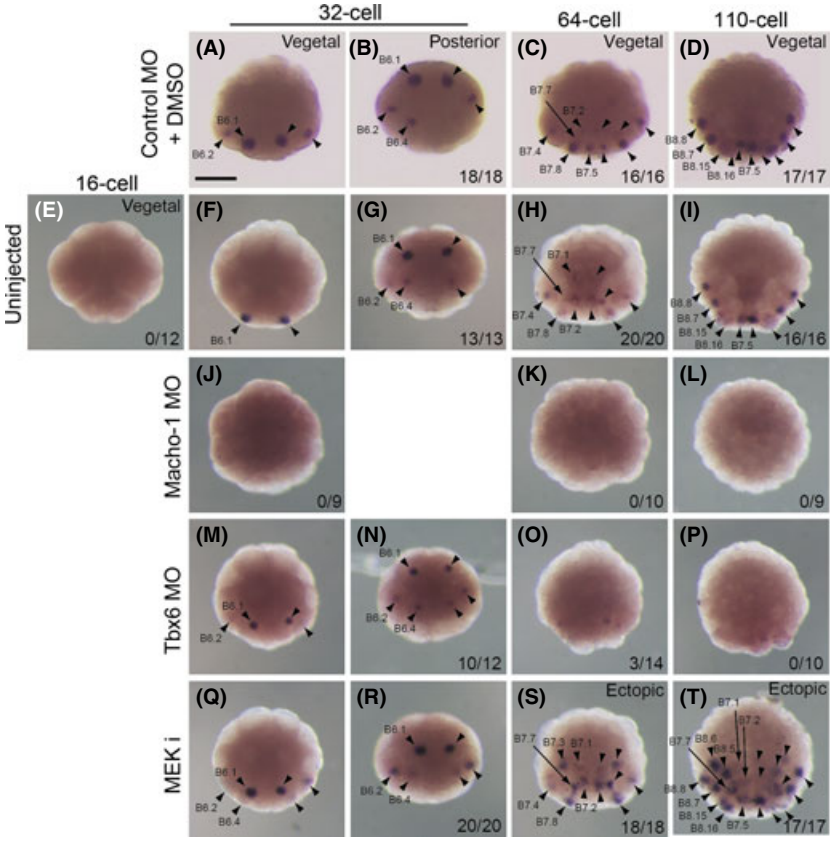

Fig. 4. (A–T) Expression of *Tbx6* revealed by *in situ* hybridization in embryos that were not treated (E–I), injected with 3 μg/μL control morpholino oligonucleotide (MO) plus treated with DMSO (dimethylsulfoxide) (A–D), injected with 3 μg/μL *Macho-1* MO (J–L), injected with 3 μg/μL *Tbx6* MO (M–P) or treated with MEK inhibitor (Q–T) in 16-cell (E), 32-cell (A,B,F,G,J,M,N,Q,R), 64-cell (C,H,K,O,S) and 110-cell (D,I,L,P,T) stage embryos. Detected signals are indicated by black arrowheads or arrows. Impaired bilateral expression was occasionally observed for unknown reasons (e.g. B6.4 in B and B6.2 in G). The numbers in the lower right-hand corners of the images indicate embryos with a similar expression pattern to control embryos, with the exception of those in (S) and (T), in which embryos showing ectopic expression were counted. Posterior views with vegetal facing upwards (B,G,N,R), and all others are vegetal views with anterior at the top (A,C–F,H,I,M,O–Q,S,T). Scale bar: 100 μm.

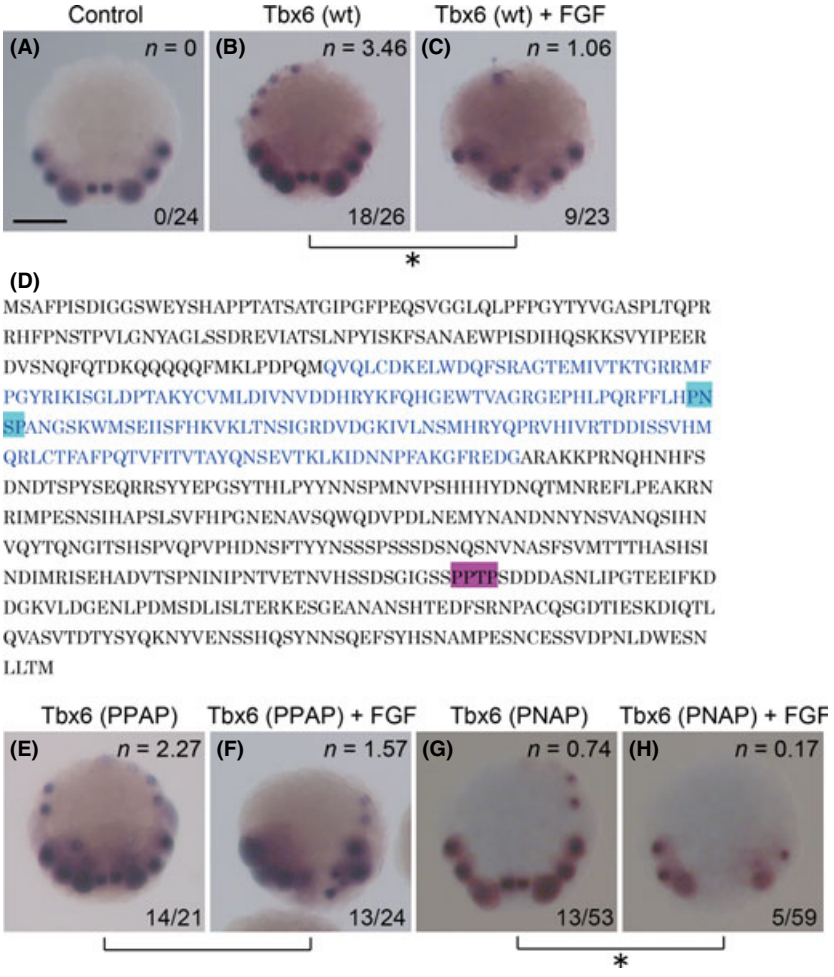

Fig. 5. (A–C,E–H) Expression of *Muscle actin* revealed by *in situ* hybridization in 110-cell stage embryos that were not injected (A), injected with 0.065 μg/μL of *Tbx6* RNA (B), injected with 0.065 μg/μL of *Tbx6*+ 0.15 μg/μL *FGF9/16/20* RNA (C), injected with 0.065 μg/μL of *Tbx6* RNA with the C-terminal mutation (PPTP>PPAP) (E), injected with 0.065 μg/μL of the mutant *Tbx6*+ 0.15 μg/μL *FGF9/16/20* RNA (F), injected with 0.065 μg/μL of *Tbx6* RNA with the T-box domain mutation (PNSP>PNAP) (G) or injected with 0.065 μg/μL of the T-box domain mutant *Tbx6*+ 0.15 μg/μL *FGF9/16/20* RNA (H). Injection of control RNA has been shown not to cause ectopic *Muscle actin* expression (Mitani *et al.* 1999). The numbers in the lower right-hand corners of the images indicate embryos with ectopic expression in nerve cord cells, whereas the numbers in the upper right-hand corners represent the average number of nerve cord cells per embryo with ectopic expression. Injection of *FGF9/16/20* RNA at the concentration used in these experiments did not cause the repression of endogenous *Muscle actin* expression. Although the image in (E) shows ectopic expression in mesenchyme precursors, it was detected only in a small percentage (19.0%) and overexpression of wild-type Tbx6 also induced it in some embryos. Scale bar: 100 μm. Asterisks indicate pairs showing significant differences by the κ^2-test. (D) Amino acid sequence of Tbx6 in *Halocynthia*. Blue letters indicate the T-box domain. The two 4-amino acid stretches in light blue and pink boxes are putative MAP kinase recognition sites and were mutated in this study to prevent their phosphorylation.

in ectopic expression in B7.3 mesenchyme precursor cells at the 64-cell stage, and B8.5 and B7.7 blastomeres at the 110-cell stage (Fig. 4S,T; compare to 4C,D,H,I). MEKi treatment also markedly increased the expression of *Tbx6* in B7.7 blastomeres at the 64-cell stage (Fig. 4S; compare to 4C,H). These results suggest that the FGF signal downregulates *Tbx6* expression in the mesenchymal lineage after segregation of the mesenchyme and muscle lineages, possibly targeting the positive feedback loop.

Phosphorylation of Tbx6 may inactivate its transcriptional activity

To determine how the FGF signal regulates *Tbx6* expression, we analyzed the effect of the FGF signal on Tbx6 transcriptional activity based on the proposed existence of the positive feedback loop. Because forced expression of *Tbx6* in the embryo leads to ectopic *Muscle actin* expression (Mitani *et al.* 1999; Fig. 5A,B), ectopic expression was used as an indicator for the

transcriptional activity. We determined the percentage of embryos with ectopic *Muscle actin* expression, which was predominantly observed in nerve cord precursor cells at the concentration of injected RNA used here (Fig. 5). Of embryos injected with wild-type *Tbx6* mRNA, 69.2% ($n = 26$) showed ectopic expression in an average number of 3.46 cells per embryo (Fig. 5B), whereas co-injection of embryos with *FGF9/16/20* and *Tbx6* mRNAs significantly reduced both the percentage of embryos (39.1%, $n = 23$; $P < 0.05$, κ^2-test) and average number of cells (1.06, $P < 0.01$, κ^2-test) expressing *Muscle actin* (Fig. 5C). These results support our hypothesis that the FGF signal inhibits Tbx6 transcription-promoting activity and the positive feedback loop in *Halocynthia* embryos.

One mechanism by which the FGF signal represses the activity of a transcription factor was demonstrated for the zebrafish T-box family member Casanova, which is inactivated upon phosphorylation by MAP kinase (Poulain *et al.* 2006), a cytoplasmic component of the FGF signaling pathway that is located downstream of MEK. We identified two possible MAP kinase recognition sites (P-Xn-S/T-P [X: neutral or basic amino acid, $n = 1$–2]; Gonzalez *et al.* 1991) in the amino acid (a.a.) sequence of Tbx6: PNSP (a.a. 225–228) within the T-box domain and PPTP (a.a. 544–547) in the C-terminal region (Fig. 5D). Therefore, we mutated the serine and threonine residues of the possible recognition sites to alanine residues to block phosphorylation, and examined the *Muscle actin* transcriptional activity of the corresponding mutants in the presence and absence of the FGF signal. Interestingly, the C-terminal mutation (PPTP>PPAP) did not significantly change the degree of ectopic expression of *Muscle actin* in the presence and absence of FGF, indicating that the mutant no longer responded to FGF (Fig. 5E,F; $P > 0.05$ for the number of cells (2.27 vs 1.57) and $P > 0.3$ for the percentage of embryos (66.7% [$n = 21$] vs 54.2% [$n = 24$]). In contrast, the transcriptional activity of the T-box domain mutant was significantly affected by the FGF signal, indicating that the mutant still responded to FGF (Fig. 5G,H; $P < 0.001$) for the number of cells (0.74 vs 0.17) and $P < 0.05$ for the percentage of embryos (24.5% [$n = 53$] vs 8.5% [$n = 59$]). Notably, the mutations alone reduced the transcriptional activity, as observed by the lower number of nerve cord precursor cells showing ectopic *Muscle actin* expression (3.46–2.27) for the C-terminal mutation (Fig. 5E), and by the reduced percentage of embryos with ectopic expression (69.2–24.5%) and number of such cells (3.46–0.74) for the T-box domain mutation (Fig. 5G). For the latter mutation, the reduction in transcriptional activity was most likely because the mutation occurred in the T-box, which is the DNA-binding domain. Taken together, these results suggest that the FGF signal may phosphorylate the threonine residue of the PPTP MAP kinase recognition site in the C-terminal region of Tbx6 via MAP kinase and thereby reduce its transcriptional activity.

Discussion

The present study provides new information concerning the spatial and temporal profiles and molecular mechanisms of muscle fate suppression in relation to mesenchyme induction. Notably, we found that mesenchyme induction requires a longer exposure to the FGF signal than that needed for muscle fate suppression. Similarly, mesenchyme induction and muscle fate suppression by MEK signaling were shown to end earlier in anterior than posterior lineages. In addition, our knockdown analyses identified the sources of the FGF signal for muscle fate suppression. Finally, we identified possible molecular mechanisms by which the mesenchyme and muscle fates are mutually suppressed. The most prominent finding in this study may reside in one such mechanism, the negative regulation of Tbx6 by FGF/MAPK-mediated phosphorylation. Based on these findings, which are consistent with what has been previously shown and proposed for mesenchyme induction, we propose an updated model for muscle suppression and mesenchyme induction.

Two-step model for mesenchyme induction

One novel finding in the study is the identification of a previously unknown source of the FGF signal for muscle fate suppression in the posterior mesenchyme B7.7 lineage, namely the B6.2 blastomere or its descendant cells (Fig. 6A). Since *FGF9/16/20* is not expressed in B6.2, the injected MO would have blocked either the translation of *FGF9/16/20* mRNA that is carried over from B5.1 or that of *FGF9/16/20* mRNA expressed in B7.4, a daughter of B6.2, at the 64-cell stage (Fig. 1F; Kumano *et al.* 2006). We consider that the involvement of B7.4 is more likely because of the late requirement of the FGF signal for posterior muscle fate suppression (Fig. 6A). In contrast, non-requirement of the B7.4 blastomere for mesenchyme induction is suggested by the finding that the recombination of B6.1 and B6.4 (posterior mesenchyme lineage) without B6.2 (B7.4 mother cell) resulted in mesenchyme induction (Kim & Nishida 1999). Although this result may have been due to the different experimental approaches used, it is also possible that when B6.4-lineage cells are recombined

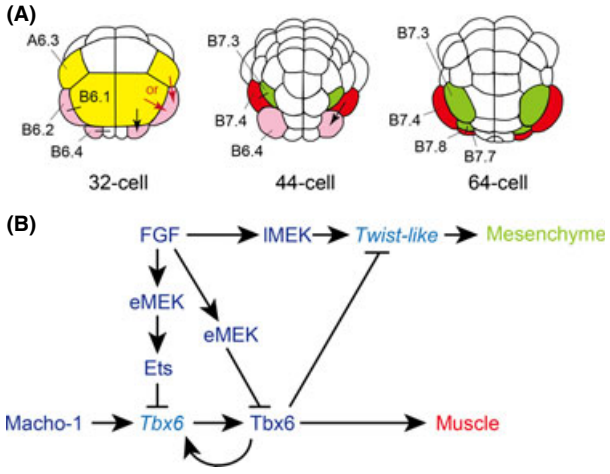

Fig. 6. Proposed model for muscle fate suppression and mesenchyme induction by fibroblast growth factor (FGF) based on data obtained from the present and previous studies. (A) Schematic diagram of mesenchyme induction in the anterior B8.5 lineage (red arrows) and posterior B7.7 lineage (black arrows). Arrows indicate the FGF signal. The signal indicated by the arrow in the 44-cell stage for B7.7 might only cause muscle suppression. The embryos at the 32-, 44- and 64-cell stages are oriented and colored in the same way as those shown in Figure 1 (A). (B) Proposed molecular model for muscle suppression and mesenchyme differentiation (see the text for details). The gene activity flow shown here is proposed to take place both in the B8.5 and B7.7 lineages, but do so in different timing as indicated in (A). Factor names in dark blue font represent proteins and those in light blue italics indicate transcripts. eMEK, early activated MEK; IMEK, late activated MEK. The inhibition of *Tbx6* transcription by Ets has not been shown experimentally.

with B6.1, they differentiate into both mesenchyme and muscle. Our results also suggest that a certain percentage of mesenchyme-lineage cells become neither muscle nor mesenchyme when embryos are treated with MEKi from the 76- or 110-cell stages. Therefore, further experiments are necessary to confirm the proposed mechanism underlying the binary fate decision between mesenchyme and muscle (Kim & Nishida 1999; Kim *et al.* 2007). In contrast to the posterior lineage, the FGF signal from either B6.1 or A6.3, but not simultaneously from both, at the 32-cell stage is sufficient for anterior muscle fate suppression (Fig. 6A), which is supported by both our local FGF inhibition experiments and previous blastomere isolation experiments (Kim & Nishida 1999).

At the molecular level, we propose that FGF9/16/20 induces mesenchyme fate by activating *Twist-like* expression and suppresses muscle formation through downregulation of the positive feedback regulation of *Tbx6* expression in a transcription-independent manner (Fig. 6B). However, we acknowledge that this transcription-independent regulation process is not the sole mechanism (Fig. 6B) as the knockdown of Ets, a transcription factor activated by the FGF/MEK/MAPK signal, was shown to induce ectopic *Muscle actin* expression in the mesenchyme lineage (Miya & Nishida 2003).

In contrast to the mesenchyme lineage, accumulation of *Tbx6* by positive feedback regulation in muscle lineage cells would likely reach the level at which the FGF signal is no longer able to suppress Tbx6 function, and would promote muscle differentiation and inhibit mesenchyme and *Twist-like* induction (Fig. 6B). This could explain why isolated mesenchyme precursor cells are no longer able to respond to the FGF signal after extended incubation in the absence of the FGF signal (Kim *et al.* 2000). Such loss of competence may be essential in the developing embryo for the late FGF signal from B7.4, which induces the mesenchyme fate in the B7.7 lineage, not to induce the mesenchyme fate in cells such as B7.4 itself that have already initiated muscle differentiation. For notochord induction, loss of competence has also been shown to be mediated by accumulation of the transcription factor FoxB, which ensures the maintenance of the default fate in environments where the FGF signal is present (Hashimoto *et al.* 2011), similar to that observed for mesenchyme induction.

Difference in the susceptible period to MEKi treatment for muscle suppression and mesenchyme induction

The present results suggest that mesenchyme induction requires later MEK activation than that for muscle fate suppression (Fig. 2A–E). This speculation is further supported by the finding that MEKi treatment from the 110-cell stage led to the reduction and nearly complete inhibition of *Twist-like* expression at the mid-gastrula stage in 84.2% of embryos (n = 19) (data not shown). Therefore, it is likely that mesenchyme induction by the MEK signal continues after the 110-cell stage while muscle fate suppression is nearly completed by the 76-cell stage. These results appear to contradict previous blastomere isolation experiments in which the percentage of isolated mesenchyme precursor cells, before induction, that developed into mesenchyme and muscle cells increased and decreased, respectively, at nearly the same timing when isolation was performed successively at different developmental stages (Kim & Nishida 1999). However, considering the fact that the isolation of cells blocks the FGF signal at the level of receptor activation and that MEKi treatment inhibits MEK activation, these contrasting results can be reconciled if mesenchyme induction and muscle suppression use different pathways, and if MEK is

activated and phosphorylates its targets earlier in muscle suppression than for mesenchyme induction (eMEK versus lMEK in Fig. 6B). This situation might differ from that in *C. savighyi* embryos, in which mesenchyme induction and muscle suppression in the posterior B7.7 lineage are both induced by transcription of *Twist-like 1* (Imai *et al.* 2003). In these embryos, knockdown of *Twist-like 1* results in ectopic *Muscle actin* expression in the mesenchyme lineage as well as elimination of mesenchyme. In contrast, in the anterior B8.5 lineage and both the anterior and posterior lineages of *C. intestinalis* embryos, muscle suppression does not proceed through the FGF-Twist-like 1 pathway (Imai *et al.* 2003; Tokuoka *et al.* 2004). Functional analysis of *Twist-like* in *Halocynthia* embryos is expected to provide insight into mechanisms by which the FGF signal suppresses muscle fate.

We also found that Tbx6 suppresses *Twist-like* expression and mesenchyme fate (Fig. 6B). This suppression pathway may ensure that the mesenchyme fate does not start to be promoted unless the activity of Tbx6, which also promotes muscle differentiation, is downregulated. There is likely a delay between the alleviation of Tbx6-mediated mesenchyme suppression and activation of *Twist-like* expression by the FGF signal, which may account for the different susceptibility periods for muscle suppression and mesenchyme induction in response to MEKi. This possibility could be confirmed by determining how Tbx6 suppresses *Twist-like* expression.

Anterior and posterior differences in susceptibility period to MEKi

The present results suggest that MEK activation is required for muscle fate suppression at later stages in posterior than anterior lineages. This finding is consistent with previous blastomere isolation experiments that demonstrated that both mesenchyme induction and muscle suppression in the posterior lineage require cell–cell contact in later developmental stages than those in the anterior lineages (Kim & Nishida 1999). The time difference for the required cell–cell contact between the anterior and posterior lineages was estimated to be between 20 and 30 min at 13°C (Kim & Nishida 1999), which is approximately the same temporal difference observed in this study between 44- and 64-, or 64- and 76-cell stages (Fig. 2C–E).

A simple explanation for the differences between the anterior and posterior lineage cells is that the former are exposed to a more intense FGF signal, as the number of inducing cells in contact with a signal-receiving cell is twice as large in the anterior (A6.3 and B6.1) as those in the posterior (B6.1) mesenchyme lineages. The timing of mesenchyme induction may also depend on the timing of *Tbx6* expression within signal-receiving cells: *Tbx6* is anteriorly expressed in B6.2 at the 32-cell stage, whereas it is posteriorly expressed in B6.4 and B7.7 at the 32- and 64-cell stages, respectively (Fig. 1G; Yasuo *et al.* 1996). Later *Tbx6* expression requires continuous exposure to the FGF signal until Tbx6 function is downregulated. Persistent *Tbx6* expression could be caused either by increased Macho-1 protein accumulation in the posterior region of the embryo due to the posterior-end localization of *Macho-1* mRNA, or by later de-repression of transcriptional regulation in posterior cells due to the presence of PEM, a germline transcriptional repressor (Kumano *et al.* 2011; Shirae-Kurabayashi *et al.* 2011), in the posterior end of the embryo. It is also possible that the difference is related to cell division progression, as the posterior-lineage B6.4 divides to produce mesenchyme-fated B7.7 approximately 20 min later than that of anterior B6.2 (Kim & Nishida 1999). If the FGF signaling cascade progresses along the time line of the cell cycle within signal-receiving cells, MEK activation could be achieved only at a certain time point during the cell cycle and thereby at different timing between cells with different cell division progression.

Acknowledgments

We are grateful to the staff members of the Asamushi Research Center for Marine Biology, the Otsuchi International Coastal Research Center, and the Seto Marine Biological Laboratory Field Science Education and Research Center for animal support. We would also like to acknowledge the *Halocynthia* genome consortium for sharing the information. This work was supported by Grants-in-Aid from MEXT Japan to G.K. and H.N.

Author contributions

G.K. designed research; G.K. performed research related to Figures 2, 3, 4 and S1; N.N. performed research related to Figures 3 and 5; G.K. and N.N. analyzed data; G.K. and H.N. wrote the paper.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Expression of *Muscle actin* revealed by *in situ* hybridization in 110-cell stage embryos that were not injected (A), injected with 3 μg/μL control morpholino oligonucleotide (MO) (B), injected with 3 μg/μL *Tbx6* MO (C) or injected with 3 μg/μL *Tbx6* MO plus 0.15 μg/μL *Tbx6* RNA (D). The numbers in the lower right-hand corners of the images indicate embryos showing expression in muscle cells at a similar level to the control. Scale bar: 100 μm.