FoxD5 mediates anterior–posterior polarity through upstream modulator Fgf signaling during zebrafish somitogenesis

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The transcription factor FoxD5 is expressed in the paraxial mesoderm of zebrafish. However, the roles of FoxD5 in anterior pre-somatic mesoderm (PSM) during somitogenesis are unknown. We knocked down FoxD5 in embryos, which resulted in defects of the newly formed somites, including loss of the striped patterns of anterior–posterior polarity genes deltaC, notch2, notch3 and EphB2a, as well as the absence of mespa expression in S-I. Also, the expression of mespb exhibited a ‘salt and pepper’ pattern, indicating that FoxD5 is necessary for somite patterning in anterior PSM. Embryos were treated with SU5402, an Fgf receptor (FGFR) inhibitor, resulting in reduction of FoxD5 expression. This finding was consistent with results obtained from Tg(hsp70l:dnfgfr1-EGFP)pd1 embryos, whose dominant-negative form of FGFR1 was produced by heat-induction. Loss of FoxD5 expression was observed in the embryos injected with fgf3/-/fgf8-double-morpholinos (MOs). Excessive FoxD5 mRNA could rescue the defective expression levels of mespa and mespb in fgf3/-/fgf8-double morphants, suggesting that Fgf signaling acts as an upstream modulator of FoxD5 during somitogenesis. We concluded that FoxD5 is required for maintaining anterior–posterior polarity within a somite and that the striped pattern of FoxD5 in anterior PSM is mainly regulated by Fgf. An Fgf-FoxD5-Mesps signaling network is therefore proposed.

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Introduction

Somite formation is defined as the reiterated subdivision of paraxial mesoderm into paired, epithelial spheres of cells on either side of the midline, and it is a common occurrence in many species. Studies reveal that there is a pre-patterning process in the anterior of the pre-somatic mesoderm (PSM) before the morphological appearance of somite pairs. Cooke and Zeeman (1976) proposed a clock and wavefront model to explain the pattern formation of PSM. They explain that a clock mechanism controls the cell oscillations between anterior and posterior somitic identities in the PSM. During this process, the position of future somite boundaries is selected in the PSM. Both anterior and posterior somitic identities are responsible for boundary formation. In fact, roles in somitogenesis have been postulated for a variety of transcription factors and signaling modulators, such as Fibroblast growth factor (Fgf), bone morphogenetic protein (BMP), T-box gene, Hedgehog (Hh), and Wnt (Nikaido et al., 2000). Based on conserved residues at distinct positions in the DNA binding domain, more than 10 different classes of Fox genes have been described. Similar to other transcription factor families, Fox genes are involved in many different developmental processes, such as mesoderm patterning during early embryogenesis. More than 100 Fox genes have been identified across species from yeasts to humans (Wijchers et al., 2006). In mice, loss of Foxa2 leads to lack of notochord and other midline structures (Weinstein et al., 1994). Foxf1 is required for lateral plate mesoderm and extra-embryonic mesoderm tissue development (Kaliničenko et al., 2001). Fox genes also play important roles during somitogenesis. FoxC1 and FoxC2 are necessary for paraxial mesodermal fate induction (Wilm et al., 2004). Foxb1- and Foxb1-deficient mice display defects in the muscle (Garry et al., 2000). In zebrafish, Odenthal and Nüsslein-Volhard (1998) classified zebrafish Fox genes and showed that the Class V Fox genes are all expressed in the somite and paraxial mesoderm, including FoxD1, FoxD3 and FoxD5. Mouse FoxD1 mutant displayed normal morphology, suggesting that FoxD1 may not be a key factor during somitogenesis (Hatini et al., 1996). Meanwhile, although it is well known that zebrafish FoxD3 mediates myf5 expression during early somitogenesis (Lee et al., 2006), the roles of FoxD5 during somitogenesis have never been reported in any species. Thus, in this report, we...
focus on the roles of zebrafish FoxD5 during somitogenesis. We found that loss of FoxD5 results in a disordered morphogenesis of somites, suggesting that FoxD5 is necessary for somite polarity. While we found that FoxD5 is regulated by both Fgf and Hh signaling, Fgf signaling appears to be the major pathway.

Materials and methods

Zebrafish husbandry and microscopy

Zebrafish were raised as described in Westerfield (1995). Mutant alleles, lia/flgb (Herzog et al., 2004) and ace/flgb (Roehl and Nüsslein-Volhard, 2001), and transgenic line Tg(hsp70:dnfgr1-EGFP)dp1, which provides the overexpression of dominant-negative FGFR1 under the control of the heat-shock-cognate-70-kd protein (hsp70) promoter (Lee et al., 2005), were used in this study. Fluorescence was visualized with a fluorescent stereomicroscope (M2 FLII, Leica) and a confocal spectral microscope (TCS SP5, Leica). The harmonics optical microscopy (HOM), which including the higher second and third harmonic generations of laser-induced fluorescence, was used to examine the structures of tissues and organs in live zebrafish embryos as it was described previously (Sun et al., 2004).

Whole-mount in situ hybridization (WISH) and immunohistochemical detection

The coding sequence of FoxD5 was isolated by RT-PCR, inserted into plasmid pGEMTeasy (Promega) and confirmed by sequencing. Riboprobes for detection of the following CDNAs were used: ch1045, deltaC, deltaD, FoxC1a, her1, her1 intron (Kawamura et al., 2005b), myf5, myod, myogenin, mespa, mespb, mbx24, notch2, notch3, paraxis, (Sawada et al., 2000), papp (Yamamoto et al., 1998) and ripply1 (Kawamura et al., 2005a). WISH and immunohistochemical detection were previously described (Lee et al., 2006), except that the hybridization method of the her1 intron probe was carried out as described by Gajewski et al. (2003). The mouse S58 anti-slow myosin antibody (Developmental Studies Hybridoma Bank) was used at a dilution of 1:10 on embryos fixed in Carnoy’s fixative and was visualized with a 1:16 dilution of donkey anti-mouse IgG-TR antibody (Santa Cruz Biotechnology). The mouse MF20 anti-myosin heavy chain antibody (Developmental Studies Hybridoma Bank) was used at a dilution of 1:10 on PFA-fixed embryos and was visualized with a 1:16 dilution of goat anti-mouse IgG-FITC antibody (Santa Cruz Biotechnology).

Injection experiments

The antisense morpholino oligonucleotides (MO) specific for the translation of mRNA for FoxD5, mbx24, flgb, flgb8, flgb8, mespa, mespb and p53 were designed to block either the translation start site or 5’-untranslated region (Gene Tools, LLC). FoxD5-MO1 (5’-GGTGATACGCTGGAGAAGCTCAT-3’), FoxD5-MO2 (5’-GGTGAGCCCTAATGTCCTGAC-3’), mbx24-MO (5’-CATTCTCCA-CACCAGACATGTCTCGG-3’), mespa-MO (5’-GACTCTACATTTAAGCTCTA-3’), mespb-MO (5’-GACGACCTCCTGCTCCTCA-3’), p53-MO (5’-GAGCCCCCTTGGACGAAATG-3’) were used. The corresponding control MOs for FoxD5-, mespa- and mespb-MO were FoxD5-5mis-MO (5’-GGTGATACGCTGGAGAAGCTCAT-3’), mespa-5mis-MO (5’-GAAacAaaaAATCAACGCTCAGAT-3’), and mespb-5mis-MO (5’-GAGACCTCCTGCTCCTCA-3’), respectively. The mismatched nucleotides of the experimental MOs are indicated by lower case. Microinjection was performed using published procedures (Westerfield, 2000).

To avoid FoxD5-MO1 binding to the synthesized FoxD5 mRNA that we microinjected for the rescue experiment, a truncated form of zebrafish FoxD5 (tFoxD5) mRNA, which lacks the sequence complementary to FoxD5-MO1, was generated by PCR and cloned into pCS2+. The mRNAs for eGFP, full-length-FoxD5 and truncated-FoxD5 were generated with the SP6 Message Machine Kit (Ambion).

Drug treatment

The drugs SU5402 and cyclopamine are widely used inhibitors of Fgf receptor (FGFR) activation (Mohammadi et al., 1997) and Hh activation (Cooper et al., 1998), respectively. SU5402 (CalBiochem) was dissolved in dimethylsulfoxide (DMSO), and cyclopamine was dissolved in 95% ethanol. Embryos were immered with either 30 μM SU5402 or 100 μM cyclopamine from the 60% epiboly-stage to bud-stage. Treatment was performed in a 3-cm Petri dish filled with 30 embryos in a total volume of 2 ml embryo medium. No effect was observed by exposing embryos to either DMSO or ethanol alone at the same concentration used for the experimental treatments. For each experiment, a portion of the embryos was collected and fixed at 12 h in 4% paraformaldehyde/PBS for WISH.

Results

Expression of the zebrafish FoxD5 gene during embryonic development

When we studied the spatiotemporal expression of FoxD5 during zebrafish embryogenesis by RT-PCR, we found that FoxD5 mRNA was maternally inherited (Fig. 1A). WISH showed that transcripts of FoxD5 were detected through the embryos, especially in the dorsal organizer during gastrulation (6 h post-fertilization (hpf); Fig. 1B). As gastrulation proceeded, a strong expression was observed at the adaxial cells flanking the future notochord, tailbud, lateral plate mesoderm (Fig. 1C) and head region (data not shown). During bud-stages, FoxD5 transcripts extended into the future head (data not shown), two stripes in the adaxial cells, two stripes in the paraxial mesoderm, and tailbud (Fig. 1D). During 12–24 hpf, FoxD5 transcripts were detected in the head region, the adaxial cells, anterior PSM and tailbud (Figs. 1E–H). During 24–36 hpf, FoxD5 transcripts were still strongly exhibited in the head region, but they were gradually reduced at the tail region (data not shown).

To determine whether the stripe expressions of FoxD5 are in the newly formed somites or in the anterior PSM, we compared the expression of FoxD5 with other genes reported to be expressed in this specific region and found that dynamic stripe expression of FoxD5 was in both the anterior PSM and newly formed somites (Figs. 1I, J). Furthermore, double ISH with FoxD5 and mespa probes revealed that FoxD5 was expressed in the S-I and S-II at 12 hpf (Fig. 1K) and co-localized with mespa transcripts. Meanwhile, double ISH with FoxD5 and her1 probes showed that FoxD5 mRNA was only expressed in the S-I at 14 hpf, and the expression domains of FoxD5 and her1 were separated and juxtaposted against each other (Fig. 1L).

Knockdown of FoxD5 boundary affected the somite formation and led to a segmentation defect

Because FoxD5 mRNA was expressed in the anterior PSM and adaxial cells, we proposed that FoxD5 might play a role in somite formation and differentiation. To study whether FoxD5 is required for somitic segmentation, we specifically blocked the translation of FoxD5 mRNA using two MOs, FoxD5-MO1 against the sequence overlapping the translation start site and FoxD5-MO2 against the 5’-untranslated sequences. The same phenotypes were observed when these two MOs were microinjected, confirming the specific defects of embryos induced by injection of MOs (Supplementary Table S1). When 1–4 ng of FoxD5-MO1 was microinjected, the morphants displayed...
Fig. 1. The spatiotemporal expression of FoxD5 during zebrafish embryogenesis. Using RT-PCR to detect the transcripts of FoxD5 during the developmental stages as indicated. Lane M was a marker, and tubulin was an internal control (A). Using WISH to reveal that FoxD5 was expressed ubiquitously at 6 hpf (B), expressed in the yolk synthetic layer and adaxial cells at 18 hpf (C), and expressed in the anterior PSM, adaxial cells and tailbud at 10–12 hpf (D) and 14 hpf (E). During 18–24 hpf, FoxD5 in the adaxial cells was down-regulated gradually (black blanket), but it was still expressed in the head and tailbud (G, H). During 12 hpf, dynamic stripe expressions of FoxD5 in the anterior PSM (S0–S-II) and newly formed somites (SI) were observed (I, J). Double ISH of mespa, her1 (labeled in red) and FoxD5 (labeled in blue) expressions during 12 and 14 hpf showed that FoxD5 was expressed in the anterior PSM from S-I to S-II (K, L).
defects in the head and trunk at 24 hpf (Figs. 2B, C). Most abnormalities were mild, including slightly reduced head size and disordered somite with an irregular boundary (Figs. 2C, F), but other abnormalities were more severe, including greatly reduced head size and distorted axis (Figs. 2B, E). Although FoxD5 morphants did not lose their somite boundaries, the arrays of paired somites became irregular (Fig. 2D vs. E, F). Moreover, the expression of somite border marker cb1045 was lost in the FoxD5 morphants (Figs. 2G, H), suggesting that FoxD5 is necessary for somite formation.

To verify the defective specificity induced by FoxD5-MO1, we co-injected with FoxD5-MO1 and a truncated form of tFoxD5 mRNA, in which the first seven amino acids of the N-terminus of FoxD5 were deleted. Under these conditions, FoxD5-MO1 specifically blocked the endogenous FoxD5 mRNA, but it failed to block the tFoxD5 mRNA we

Fig. 2. Loss of FoxD5 function resulted in loss of somite polarity during somitogenesis. The FoxD5-MO1-injected embryos with abnormal phenotypes were categorized as having severe defects, such as smaller head size and distortion axis (B), and mild defects, such as reduced head and disordered somites with irregular boundary (C). Dorsal views of trunk somites in WT embryos (WT; D) and FoxD5-MO1-injected embryos (E, F) at 18 hpf. In WT embryos, column-shaped, maturely formed somites (indicated by black arrowheads) and two newly forming somites S-I and S-II (blue arrowheads) were observed. Meanwhile, in FoxD5 morphants, wider and more raggedly formed mature somites (black arrowheads) and newly forming somites, but with irregular somitic furrows (red arrowhead), were observed. The expressions of cb1045 at 18-hpf in WT embryos and FoxD5 morphants were also studied. The cb1045 was specifically observed in the somite borders of WT embryos (G), but it was lost in FoxD5 morphants (H). Co-injection of FoxD5-MO1 and tFoxD5 mRNA enabled embryos to rescue the defective expression of cb1045 induced by FoxD5-MO1 alone (I). In vivo HOM sectioning of WT embryos (J–L) and FoxD5 morphants (M–O) at 24 hpf was examined under bright field (J, M) and dark field microscopes (K, L, N, O). The morphological structures, including skin and somite boundaries, which are labeled in purple, were observed by third harmonic generation signal, whereas muscle fibers, which are labeled in green, were observed by second harmonic generation emissions. Compared to the WT embryos (K, L), the structure of the somites in the FoxD5 morphants was disordered (N, O).
microinjected. We noticed that the tFoxD5 mRNA should be as functional as the full-length of FoxD5 mRNA because no significant difference of defects was observed between embryos injected with the tFoxD5 mRNA and FoxD5 mRNA (Supplementary Table S2). In the rescue experiment, the defective phenotype caused by FoxD5-MO1 was not rescued by co-injection of egfp mRNA with FoxD5-MO1 (Supplementary Table S1). However, embryos displaying the normal phenotype dramatically increased when the tFoxD5 mRNA was co-injected with FoxD5-MO1 (Supplementary Table S1, Figs. S1D, D’). When we microinjected 4–8 ng of control FoxD5-5mis-MO, which contains five mismatched nucleotides against FoxD5-MO1, no phenotype was observed (Supplementary Table S1; Figs. S1E, E’). Similarly, the number of embryos displaying the normal phenotype dramatically increased when full-length FoxD5 mRNA was co-injected with FoxD5-MO2 (Supplementary Table S1). Moreover, the excessive tFoxD5 mRNA enabled embryos to rescue the disordered somite boundary caused by FoxD5-MO1, and these embryos were able to regain cb1045 expression (Fig. 2I). Besides, to rule out the possibility that the defects in FoxD5 morphants were caused by the toxicity of MOs, we co-injected 3 ng of FoxD5-MO1 and 6 ng of p53-MO. Results showed that the somatic boundaries were not rescued, but instead were still disorganized (Fig. S1C), indicating that the structural defects in somite and the reduction of cb1045 expression (Fig. 2H vs. 1) in FoxD5-MO1-injected embryos were specific and dependent upon FoxD5 loss of function.

To understand which kind of somite defects occurred in the FoxD5 morphants, we used in vivo sectioning to analyze the somite development of wild-type (WT) embryos and FoxD5 morphants by HOM and laser-induced fluorescence higher harmonic generations. The epidermis, somite boundary and notochord were clearly identified by third harmonic generation signals (purple labels in Figs. 2K, L, N, O). The myotomes, which are composed of collaterally organized myosin and actin filaments, appeared strongly by second harmonic generation emissions as a result of their highly organized nano-structures (green labels in Figs. 2K, L, N, O). The anterior/posterior identity of cells in the anterior PSM was impeded by third harmonic generation signals (purple labels in Figs. 2K, L, N, O). The myotomes, which are composed of collaterally organized myosin and actin filaments, appeared strongly by second harmonic generation emissions as a result of their highly organized nano-structures (green labels in Figs. 2K, L, N, O). The trunk and tail somites of WT embryos at 24 hpf were exhibited distinctly (Figs. 2K, L). However, the trunk somite in the FoxD5 morphants showed incomplete development (Figs. 2M, N), while the tail somite displayed a severely disordered structure. Abnormally disorganized structures were observed in the tail somites (Fig. 2O), indicating that the development of somites is impeded in FoxD5 morphants. In addition, the abnormal morphology of somite structure in FoxD5 morphants suggested that the segmental patterning is disturbed as well. Thus, we proposed that the stripe expression of FoxD5 in the anterior PSM is required for normal formation of somite in furrow.

The establishment of segmental pre-pattern was processed normally in the FoxD5-knockdown embryos

Before epithelial somites are formed, precise anterior and posterior domains are established in the somite primordial. It has been reported that Notch-dependent signaling is required during this step (Johnson et al. 2000; Sawada et al. 2000; Ozbudak and Pourquié, 2008). The oscillatory behavior in the PSM is manifested in the zebrafish embryo by periodic waves of expressions of deltaC and her1 that appear to extend anteriorly from the tail bud (Jiang et al. 2000; Goldbeter and Pourquié, 2008; Holley et al., 2000). In this study, we found that the expression patterns of deltaC and her1 in the PSM of FoxD5-MO1-injected embryos were presented as a dynamic scheme of periodically changing broad and narrow strips, which were similar to those in the PSM of WT embryos (Figs. 3A, C vs. B, D). Similar to the WT embryos, the dynamic expression of her1 in the PSM of the FoxD5-MO1-injected embryos was also normal (Fig. 3B vs. F). In addition, the cyclical expression of the her1 intron probe was also detected in the WT embryos and the FoxD5 morphants (data not shown). Thus, knockdown of the FoxD5 gene in zebrafish embryos did not affect gene oscillation in the posterior PSM. However, unlike the deltaC expression in the somite of WT embryos, the striped expression of deltaC was not sustained in the somites of FoxD5-MO1-injected embryos during 10–14 hpf (Figs. 3B, D). These evidences indicate that the first step of segmentation such as the establishment of segmental pre-pattern is not affected by FoxD5 inhibition, but rather, that synchronized oscillatory behavior does occur in the PSM of FoxD5-MO1-injected embryos. Thus, the disorganized structure of somites in FoxD5 morphants might be because FoxD5 expression is absent in the anterior PSM, not because FoxD5 is absent in tailbud and posterior PSM.

The anterior–posterior polarity in somites was lost in the FoxD5 morphants

The reduced deltaC expression in the somites of FoxD5 morphants suggested that the anterior–posterior polarity in the somites might be affected. We examined the expressions of notch3 and notch2, which label anterior and posterior somites, respectively. We found that expressions of notch2 (Fig. 3G) and notch3 (Fig. 3I) appeared in striped pattern in the somites of WT embryos. However, instead of a striped pattern presented in the somites of WT, a smeared signal was observed in this same region of the FoxD5-MO1-injected embryos (Figs. 3H, J). These results suggest that FoxD5 is necessary for normal somitogenesis and that FoxD5 functions in controlling the anterior–posterior polarity of somites.

Since the ectopic expression of Eph/Ephrin signaling leads to abnormal somitogenesis (Durbin et al. 1998), we examined the expression of ephrinB2a in FoxD5 morphants. Results showed that the striped pattern of ephrinB2a was lost, and a smeared pattern throughout the somitic region in FoxD5 morphants was observed (Fig. 3K vs. L). Mice lacking paraxis do not form somite epithelium and show severe defects of muscle (Burgess et al., 1995; 1996). Here, however, it is the striped pattern of paraxis that was lost in the somites of FoxD5-knockdown embryos (Fig. 3M vs. N). The paraxial protocadherin, pacp, a member of the cadherin superfamily, is an important regulator during somitogenesis (Yamamoto et al., 1998). Interestingly, while pacp expression domains located at S-I and S-II were still present in MO-injected embryos (Fig. 3O), the expression of mespa (Fig. 3Q) appeared in the anterior PSM of FoxD5 morphants (data not shown). This indicated that the expression of mespa in the FoxD5 morphants might be because pacp expression in the anterior PSM when somites start to form. Taken together, the effective expressions of the genes we studied in the somites of FoxD5 morphants indicated that FoxD5 is an essential factor for anterior–posterior patterning during somite furrow formation.

The anterior/posterior identity of cells in the anterior PSM was impeded in FoxD5-MO1-injected embryos

Next, we focused on the anterior PSM where FoxD5 is expressed in striped pattern. The mesp genes play a conserved role during segmental patterning of the mesoderm in vertebrate embryos by specifying segmental boundaries and anterior–posterior segmental polarity (Sawada et al., 2000). Previous studies (Takahashi et al., 2000) revealed that mesp genes specifically affect Notch signaling in the anterior PSM. In this study, we found that both mespa and mespb genes were expressed in overlapping domains in the anterior of the somite primordia S-I and S-II during somitogenesis in zebrafish embryos (Figs. 4A, C), as Durbin et al. (2000) and Sawada et al. (2000) reported. In addition, a transient band of mespb expression was observed in the forming somite S0 (Fig. 4C). However, we observed that the expression of mespa in S-I was lost in the FoxD5-MO1-injected embryos (Fig. 4B). In contrast, while mespb expression lost its...
striped pattern, it appeared as a “salt and pepper” pattern in the FoxD5-deficient embryos (Fig. 4D). Therefore, even though FoxD5 affects mespa and mespb differentially, it is still required for normal expression of these two genes in the anterior PSM.

**FoxD5 did not function in the segmental pre-pattern**

Zebrafish T-box transcription factor Tbx24 is necessary for somite formation (van Eeden et al., 1996; Nikaido et al., 2002). In WT embryos, tbx24 was expressed in the PSM and in the anterior part of forming somites S0 to SII (Fig. 4E). Although tbx24 expression in the PSM of FoxD5 morphants was as same as that of WT embryos, tbx24 failed to maintain segmented expression in somites when FoxD5 was absent (Fig. 4F). In other words, FoxD5 was still expressed in the tailbud and adaxial cells in the tbx24 morphants, but not in the anterior PSM, specifically, S0 to S-II (Fig. 4G vs. H). Therefore, we suggested that FoxD5 might be downstream of tbx24 and that, therefore, FoxD5 plays a role in the anterior PSM to maintain somitic anterior–posterior polarity, but not segmental pre-pattern.

**The modulation of Fgf and Hh signaling on FoxD5 expression**

Next, we attempted to study which signaling is necessary for FoxD5 expression during somite formation in zebrafish embryos. The Fgf signaling pathway in the PSM controls cell maturation and the positioning of segmental boundary (Sawada et al., 2001; Dubrulle and Pourquié, 2004; Wahl et al., 2007). To investigate the temporal requirement for Fgf signaling, we immersed embryos in embryo medium containing SU5402, an FGFR inhibitor. The SU5402-treated embryos were incubated, starting at 60% epiboly, until 6–8 somite-stages. To demonstrate the effectiveness of the treatment, a subset of the embryos was fixed immediately and processed for WISH of FoxD5. In all SU5402-treated embryos, the FoxD5 transcripts were barely detectable in the anterior PSM (Fig. 5B); however, we could still observe the somite structure (data not shown), although the myod signals were greatly reduced (Fig. 5I). Moreover, embryos derived from outcrossing a homozygous strain of Tg(hsp70L:dnfgfr1-EGFP);pd1 fish were treated with heat shock induction at 37 °C for 1 h at 8–9 hpf, with analysis of the FoxD5 transcripts during 12 hpf. Although we
observed somite formation in these heat shock-treated embryos, WISH of the FoxD5 transcripts revealed that inhibition of Fgf signaling greatly reduced FoxD5 expression (Fig. 5C). When embryos were treated with SU5402 for 4 h, starting at 12 hpf, with analysis of the FoxD5 transcripts during 16 hpf, we found that FoxD5 expression was still greatly reduced (data not shown). Additionally, when embryos derived from Tg[hsp70l:dnfgr1-EGFP]pd1 were treated with heat shock induction at 10–11 hpf, with analysis of the FoxD5 transcripts during 12 hpf, we observed that these embryos continued to display a reduction of FoxD5 transcripts (data not shown). These findings strongly suggested that the striped expression of FoxD5 in the anterior PSM is dependent on Fgf activity. We also noticed that the fgf8 and fgf3 mutants showed slightly reduced expressions of FoxD5 in the adaxial cells and anterior PSM (Figs. 5D, E). However, fgf3-/fgf8-double morphants showed that the expression of FoxD5 was almost lost in the adaxial cells and the anterior PSM at 12 hpf (Fig. 5F), suggesting that Fgf3 and Fgf8 might have redundant function in regulating FoxD5 expression. Taken together, we proposed that Fgf signaling is an upstream modulator of FoxD5 during somitogenesis.

Hh signaling has been shown to promote myogenesis in both fish and amniotes (Ingham and McMahon, 2001; Borello et al., 2006). After we treated embryos with the Hh inhibitor cyclopamine, starting at 60% epiboly, 80% epiboly or bud-stages, and ending at 6–8 somite-stages, we performed WISH which showed that FoxD5 was detected in the somites and anterior PSM, but was lost in the adaxial cells in cyclopamine-treated embryos (Fig. 6A vs. F). A slightly enhanced and extended expression of FoxD5 in the anterior PSM was observed in cyclopamine-treated embryos (Fig. 6A vs. F), suggesting that Hh signaling is necessary to inhibit FoxD5 expression in the anterior PSM. In addition, the ectopic expression of FoxD5 did not cause the ectopic expressions of mespa and mespb (Figs. 6C, D vs. H, I). These results suggest that some other type of signaling represses the expression of mesps, such as ripple1, and may still be present in the somites of cyclopamine-treated embryos (Fig. 6E vs. J). On the other hand, myod transcripts were only detected in the somites, but not in the adaxial cells (Fig. 6B vs. G). Thus, the loss of FoxD5 and Myod in the adaxial cells of cyclopamine-treated embryos was the result of incomplete development of the adaxial cell structure. Therefore, we concluded that Hh activity is required for inhibition, rather than induction, of FoxD5 expression.

Excessive FoxD5 mRNA could rescue the defective expression levels, but not the striped patterns, of mespa and mespb in the absence of Fgf signaling.

We have demonstrated that FoxD5 plays roles in modulating mespa and mespb expressions and that FoxD5 is regulated by Fgf signaling. However, precisely which factor or signaling is required for the expression of mesps during somitogenesis is unclear. To define further the molecular network among Fgf, FoxD5 and Mesps during zebrafish somitogenesis, we analyzed the expressions of mespa and mespb in the fgf3 mutant, the fgf8 mutant and the fgf3-/fgf8-double-MO-injected embryos. Results showed that two stripes of mespa expression in the anterior PSM were slightly reduced in both fgf3 and fgf8 mutants (Fig. 7A vs. C, E), while mespa was greatly reduced in the fgf3-/fgf8-double morphants (Fig. 7I). Interestingly, the pattern of mespa expression in the fgf8 mutant was similar to that which appeared in the FoxD5-MO1 morphants (Fig. 4B vs. Fig. 8A). That is, two or three stripes of mespb in the anterior PSM were slightly reduced in both the fgf3 and fgf8 mutants (Fig. 7B vs. D, F), but greatly reduced in the fgf3-/fgf8-double morphants (Fig. 7I). In embryos derived from the transgenic line Tg[hsp70l:dnfgr1-EGFP]pd1 and treated with heat-shock induction at 10–11 hpf, it was found that both mespa and mespb at 12 hpf were greatly reduced (Figs. 7G, H). Furthermore, when FoxD5 mRNA was co-injected with fgf3-/fgf8-double-MOs, the expression levels of mespa and mespb were nicely rescued (Figs. 7L, J vs. K, M). However, we noticed that the stripe patterns of mespa and mespb were not restored by injection of FoxD5 mRNA in the fgf3-/fgf8-double morphants. Thus, we hypothesized that some factor, other than FoxD5, might be the target of Fgf signaling in regulating the striped expression of mesps.

Fig. 4. Effect of inhibiting FoxD5 protein synthesis on the expression of mespa and mespb. By WISH, the expressions of mespa, mespb and papc in wild-type embryos (WT) and FoxD5 morphants (FoxD5 MO) were observed at 12–24 hpf as indicated. mespa was expressed at somites S-I and S-II in WT (A), but was greatly reduced in FoxD5 morphants (B). mespb was detected from S0 to S-II as a striped pattern in WT (C), but failed to maintain a striped expression (D). Expression of tbx24 was detected in the PSM and the anterior part of formed somites from S0 to S-II in WT (arrowheads in E). Although tbx24 expression was unchanged in the PSM, it failed to maintain a segmented expression at the somite region in the FoxD5 morphants (arrows in F). FoxD5 was expressed at tailbud, adaxial cells, and forming somites in WT (arrowheads in G), whereas FoxD5 was still expressed at tailbud and adaxial cells, but was not at the forming somite region in the tbx24 morphants (H).
The forkhead transcription factor FoxC1a plays important roles during early somitogenesis in zebrafish (Topczewska et al., 2001). Therefore, we examined FoxC1a to see whether any factor other than FoxD5 could be the target of Fgf signaling in regulating the striped expression of mesps. Interestingly, when FoxC1a was knocked down, mespa expression remained unchanged (Fig. 7M), whereas mespb expression was almost absent (Fig. 7N). We then examined the expression of FoxC1a in fgf3-/fgf8-double-MO-injected embryos and observed that the expression of FoxC1a was strongly reduced in the fgf3-/fgf8-double morphants (Supplementary Fig. S3). Therefore, we proposed that FGF might mediate FoxC1a, which is not dependent on FoxD5, to control mesp gene expression.

Holley et al. (2000) and Oates et al. (2005b) reported that fss/tbx24 may function primarily in a cell-autonomous manner to direct the anterior half-somite fate, which then induces or promotes the adoption of posterior half-somite fate in the neighboring cells. Moreover, in the tbx24/fss mutant, mespa, mespb, fgf8, notch3 and papc almost lost their expressions (Oates et al., 2005b). To more clearly understand the relationship between tbx24 and FoxD5 and to distinguish whether FoxD5 functions in the anterior PSM under the control of tbx24, we carried out the rescue experiment of tbx24 morphants with injection of excessive FoxD5 mRNA (Fig. 8; Supplementary Table S3). We demonstrated that the somite structure (Fig. 8A) and the striped expression of myod (Fig. 8D) were normal in WT embryos. However, in the tbx24-MO-injected embryos, the somite structure (Fig. 8B) and the segmental expression of myod (Fig. 8E) were lost. Instead, the myod transcript was uniformly expressed throughout the somitic region of the paraxial mesoderm (Fig. 8E). When embryos were co-injected with tbx24-MO and FoxD5 mRNA, neither the somitic defect (Fig. 8C) nor the myod pattern defect (Fig. 8F) was rescued. In addition, we analyzed the expressions of mespa and mespb to see whether these somitic polarity genes could be rescued by injection of FoxD5 mRNA in the tbx24-MO-injected embryos. Results showed that the transcripts of mespa (82 out of 94 embryos examined; Fig. 8G vs. H) and mespb (63 out of 74 embryos examined; Fig. 8K vs. L) in the tbx24 morphants at 12 hpf were almost lost, a result which is consistent with what Sawada et al. (2000) reported. Surprisingly, we noticed that about 13–15% of tbx24 morphants still showed uniform expressions of mespa (12 out of 94 embryos examined; Fig. 8G vs. H) and mespb (63 out of 74 embryos examined; Fig. 8K vs. L) throughout the anterior PSM region. However, when embryos were co-injected with tbx24-MO and FoxD5 mRNA, all the injected embryos lost mespa (Fig. 8J) and mespb (Fig. 8N) transcripts during 12 hpf, suggesting that excessive FoxD5 mRNA...
did not enable embryos to rescue the defects caused by tbx24-MO. Thus, FoxD5 was not able to induce mespa and mespb expressions in the absence of tbx24.

Discussion

In this study, we investigated a forkhead gene, FoxD5, which controls somite morphogenesis. We conclude that (1) the expression of FoxD5 in the anterior PSM is necessary for anterior–posterior polarity maintenance; (2) Fgf signaling is a major upstream modulator of FoxD5 expression in the anterior PSM to regulate the anterior–posterior polarity during somite formation; and (3) Fgf-FoxD5-Mesps represents a novel molecular network model for somitogenesis in zebrafish (Fig. 9).

Zebrafish FoxD5 is a downstream target of tbx24 during segmentation

Many studies reveal that tbx24 is necessary for segmental pre-patterning to initiate the segmentation oscillator in the segment polarity zone (van Eeden et al., 1996; Durbin et al., 2000; Holley et al., 2000; Nikaido et al., 2002; Oates et al., 2005b). In the tbx24/fss mutant, mespa, mespb, fgf8, notch3 and papc nearly lose their expressions (Oates et al., 2005b). In this study, tbx24 morphants exhibit a normal expression of FoxD5 in the adaxial cells, but the striped pattern in the anterior PSM is lost (Fig. 4G vs. H). While these findings suggest that FoxD5 may not be involved in segmental pre-patterning, they do point to FoxD5 as a possible downstream factor of tbx24.

On the other hand, the expression of tbx24 in the PSM is unaffected in FoxD5 morphants, while the striped pattern of tbx24 in somites is disordered (Fig. 4E vs. F). Using the genetic mosaic experiment, Holley et al. (2000) and Oates et al. (2005b) reported that fss/tbx24 may function primarily in a cell-autonomous manner to direct the anterior half-somite fate, which then induces or promotes the adoption of posterior half-somite fate in the neighboring cells. Thus, the remainder of disorganized tbx24 transcripts in the newly formed somite cells of the FoxD5 morphants may cause the chaotic expression of some genes, such as fgf8 (data not shown) and notch (Fig. 3) in the neighboring cells. Moreover, the striped pattern of tbx24 in somites is disordered in FoxD5 morphants (Fig. 4E vs. F), reveals that there is a feedback control by which FoxD5 also controls the stripe expression of tbx24. Taken together, we proposed that the expression of FoxD5 is necessary in the fss/tbx24-expressing cells, whose fates have been determined, and that FoxD5 controls the somite polarity-associated gene expression, thus completing segmental polarization.

The relationship between muscle differentiation and myotome boundary formation is complex. The tbx24/fss mutant loses somite structure, but its muscle cell differentiation appears normal (van Eeden et al., 1996; Nikaido et al., 2002), indicating that the somite boundary formation is not dependent on cell differentiation during segmental patterning. However, there are actually two studies indicating that muscle differentiation enables embryos to rescue myotome boundary formation in the segmentation mutant (van Eeden et al., 1998; Henry et al., 2005). In this study, knockdown of FoxD5 disrupts normal somite boundary formation, but the muscle differentiation is not affected. Although the expression of myf5 in the somite of FoxD5-deficient embryos is lost, other MRFs, such as myod and myogenin, are still expressed (Supplementary Fig. S4). Moreover, the muscle fibers are detected using MF20 antibody (Supplementary Fig. S5). This line of evidence indicates that the differentiation of anterior PSM cells is processed. We also notice that the striped expressions of myod (Fig. S4D; n = 77, 83%) and myogenin (Fig. S4F; n = 94, 87%) are affected in FoxD5 morphants. The transcripts of myod and myogenin, whose signals from the posterior part of somites are extended to the anterior part of somites, are abnormally expressed (Fig. S4). This fact may be a result from the disordered anterior–posterior polarity within the somites of FoxD5 morphants, which, in turn, accounts for the misalignment of myofibrils (Fig. S5).

FoxD5 functions as an upstream modulator to regulate mespa and mespb differentially during somitogenesis

Zebrafish mespa and mespb belong to the Mesp-related subfamily of bHLH transcription factors, but they are not one-to-one orthologs of...
the murine Mesp1 and Mesp2 gene (Saga et al., 1996 and 1997; Sawada et al., 2000). Mesp1 plays an important role during early cardiac mesoderm lineage specification (Saga et al., 1999; Wu, 2008). However, the role of Mesp1 during somitogenesis is little studied. Mesp2 seems to play major roles in somitogenesis. Mesp2-deicient mice showed a disturbance of the somitic antero-posterior polarity during somitogenesis. Moreover, no paraxial mesoderm is generated in the Mesp1/Mesp2-double-knockout embryo, which results in a complete lack of somites (Saga et al., 2000). Therefore, these two Mesp isoforms are necessary for somite formation.

In zebrafish, gain-of-function studies reveal that ectopic expression of mespa causes a severe gastrulation defect, probably caused by the inhibition of mesoderm formation. Ectopic expression of mespb causes a loss of the posterior identity within the somite primordium (Sawada et al., 2000). In this report, knockdown of FoxD5 greatly reduces the mespa expression in S-I during somitogenesis, but all
FoxD5 morphants exhibit normal mespa expression during gastrulation, suggesting that mespa and FoxD5 play out their roles in different pathways during gastrulation. Specifically, our data demonstrated a differential requirement of FoxD5 protein for expressions of mespa and mespb. While the expression of mespa is greatly reduced in FoxD5-knockdown embryos in S-I (Fig. 4B), the expression of mespb loses its striped pattern and shows a “salt and pepper” pattern (Fig. 4D). In other mutants, such as bea, des, aei and mib, the mespb expression pattern shows a mosaic “salt and pepper” pattern. Here, we notice that the “salt and pepper” pattern of mespb expression in FoxD5 morphants is not as disorganized as seen in the Notch mutants, suggesting that the factor causing the “salt and pepper” pattern of mespb expression in FoxD5 morphants may not be the same as that affecting Notch mutants. On the other hand, mespa expression in these mutants tends to be very weak and slightly diffuse, which is different from that of FoxD5 morphants which only lost mespa expression in S-I (Fig. 4; Sawada et al., 2000). We therefore proposed that FoxD5 directs mespa expression in S-I and that the disordered expression of mespb probably results from an indirect effect caused by irregular segmentation. This evidence also reveals that mespa and mespb are differentially regulated. The same differential effect on mesp expression has also been observed in FoxC1a morphants.
The role of FoxD5 in the anterior PSM to maintain the anterior–posterior polarity identity

The Notch signaling pathway is critically necessary for somite development. Mesp2 plays important roles in somite anterior–posterior patterning through interaction with Notch/Delta signaling (Takahashi et al. 2000). We concluded that the activities of both mespa and mespb are required for guiding the anterior–posterior patterning of the presumptive somites and that the defects in the anterior PSM are accompanied by the absence of the striped expression of notch2 and notch3 genes in the mature somites of FoxD5 morphants (Fig. 3). We noticed that the expression level of deltalC is greatly reduced in the somites of FoxD5 morphants (Fig. 3C vs. D). The decrease of deltalC may result in abnormal segmental patterns of notch2 and notch3 in the somites, which are similar to those of the phenotypes induced by deltalC-MO, as reported by Oates et al. (2005a). Thus, FoxD5 is necessary for the normal patterns of mesp and notch genes, indicating that the role of FoxD5 involves anterior–posterior patterning. Although the Notch gene in the somite is affected when FoxD5 is knocked down, the oscillation of the Notch gene in the PSM is normal. Moreover, by measuring the length between the tailbud and anterior PSM of embryos co-injected with fgf3/-/fgf8-double-MO and FoxD5 mRNA, we find that excessive FoxD5 mRNA enables embryos to rescue the defective expression of mesp, but it does not enable rescue of either gene expression in the posterior PSM or the maturation state of the PSM (data not shown). Taken together, we proposed that the absence of FoxD5 protein does not influence the oscillation of cells in the PSM.

On the other hand, our data demonstrate that excessive FoxD5 mRNA can enhance mesp gene expression only in the anterior PSM of the fgf3/-/fgf8-MO-injected embryos, even though the injected FoxD5 mRNA is distributed ubiquitously. Moreover, our data also show that excessive FoxD5 mRNA does not enable rescue of the decreased expression of mesp genes induced by the knockdown of tbx24 (Fig. 8). These evidence indicate that FoxD5 is not able to induce either mespa or mespb expression in the absence of tbx24. Many studies have proven that tbx24 is primarily cell-autonomous to direct the anterior half-somite fate, which then induces or promotes the adoption of posterior fates in neighboring cells (Holley et al., 2000; Oates et al., 2005b; Holley, 2007). Based on this evidence, we proposed the existence of an fss/tbx24-dependent, cell–cell signaling in the anterior PSM which helps FoxD5 induce the expression of mespa and mespb. FoxD5 is not able to activate mesp genes alone, suggesting that FoxD5 is required for the maintenance of mesp expression.

Fgf-FoxD5-Mesp signaling network controls somite formation

During somite formation, Fgf signaling plays important roles in regulating the differentiation of PSM cells along the anterior–posterior axis from a state which permits oscillating gene expression to a state which drives the segmentation processes. Specifically, while intensity gradients of Fgf signaling in the PSM maintain cells of the posterior PSM in an immature state, these same gradient gradients also control the maturation wavefront in anterior PSM (Goldbeter and Pourquié, 2008). MET is also controlled by the level of Fgf signaling in the PSM, and the MET process begins once the cells enter the anterior PSM, which is characterized by lower Fgf signaling (Dubrulle et al., 2001; Delfini et al., 2005). In the anterior PSM of chick and mouse embryos, the beginning of the epithelialization process correlates with the down-regulation of Snai1 and Snai2 and is mediated by Fgf signaling (Selton et al., 1998; Dale et al., 2006). Therefore, Fgf signaling is not only involved in oscillating gene expression in the PSM, but also the MET process during somite formation. Moreover, no matter whether we inhibit FGFR or knock down Fgf ligands, the expression of FoxD5 is inhibited significantly, suggesting that Fgf signaling directs FoxD5 expression during somitogenesis. Additionally, both double knockdown of fgf3/fgf8 and inhibition of FGFR cause the loss of almost all the FoxD5 transcripts. These results indicate that Fgf factors might play a redundant function in mediating FoxD5 expression. Our data also demonstrated that Fgf signaling is necessary for the expression of mespa and mespb. Therefore, we propose a de novo Fgf-FoxD5-Mesps signaling cascade during somitogenesis.

Although the expression levels of mespa and mespb are greatly rescued by FoxD5 mRNA in the fgf3/-/fgf8-double-MO-injected embryos, we notice again that the striped patterns of mespa and mespb are not restored in the absence of Fgf signaling. Furthermore, FoxC1a is strongly reduced in the fgf3/-/fgf8-double-MO-injected embryos (Supplementary Fig. S3). Knockdown of FoxC1a loses the expression of mespb during early somitogenesis (Fig. 7P). Moreover, Wahl et al. (2007) proved that Fgf acts upstream of the Wnt and Notch pathways to control the segmentation clock oscillations. Therefore, it

Fig. 9. A plausible model representing the modulation of FoxD5 during somitogenesis in zebrafish. Schematic illustration of a signaling network model for somitogenesis in zebrafish embryos. Regulated by Tbx24 and Fgf signaling, FoxD5, which is conformationally expressed in the anterior PSM, ultimately controls the differential expressions of mesps which, in turn, regulate somite polarity. FoxD5 also inhibits papc and maintains the striped pattern of paraxis to control the MET processes during somite formation.

(Topczewska et al., 2001) and ripply1 morphants (Kawamura et al., 2005a).

Interestingly, in our study, knockdown of either mespa or mespb in zebrafish causes a defect in somite boundary formation (Supplementary Table S1; Fig. S6), indicating the importance of mesp in somite formation. We also show that mesps do not represent upstream modulators of FoxD5, particularly since normal expression of FoxD5 is observed in mespa and mespb morphants (Fig. S6).
is plausible that Fgf might mediate the Wnt and Notch pathways, which are independent of FoxD5, to control somite formation. This evidence indicates that the entire Fgf-mediated regulatory network during somite formation is a complex process, which leads to the speculation that Fgf might regulate somite formation in a multi-signaling manner, not exclusively through FoxD5.

Another possibility is that the ubiquitous, but not stripy, expression of FoxD5 mRNA in fgf3-/fgf8-double morphants might explain why injection of FoxD5 mRNA does not help at all to recover the stripy expression of the mespa genes. That is, the expression of mespa genes cannot be completely rescued by providing FoxD5 mRNA everywhere in the fgf3-/fgf8-double morphants.

The expression of FoxD5 in the anterior PSM is confirmed by Hh signaling

Hh signaling plays important roles during somitogenesis (Borello et al., 2006). When cyclopamine interferes with Hh signaling, development of adaxial cells is inhibited (Fig. 7). Interestingly, the ectopic expression of FoxD5 in the anterior PSM and the extension of striped pattern from S-2 to S-4 are observed in Hh-deficient embryos. These results suggest that Hh signaling is necessary to confine FoxD5 expression to the anterior PSM. Although the FoxD5 transcripts are ectopically expressed in somites, neither mespa nor mespb is extended into somites. This might result from the fact that the repressor ripply1 is still present in the somites of FoxD5 morphants, which results in confinement of the expressions of mespa and mespb in somites (Fig. 6). Additionally, although most genes that are expressed in anterior PSM and involved in somite segmentation are repressed by ripply1, these results also reveal that FoxD5 is not repressed by ripply1 (Kawamura et al., 2005a).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ydbio.2009.10.001.

References


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