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Mesogenin causes embryonic mesoderm progenitors to differentiate during development of zebrafish tail somites

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ABSTRACT

The molecular mechanism underlying somite development differs along the embryonic antero-posterior axis. In zebrafish, cell lineage tracing and genetic analysis have revealed a difference in somite development between the trunk and tail. For instance, *spadetail*/*tbx16* (*spt*) mutant embryos lack trunk somites but not tail ones. Trunk and tail somites are developed from mesodermal progenitor cells (MPCs) located in the tailbud. While the undifferentiated state of MPCs is maintained by mutual activation between Wnt and Brachyury/Ntl, the mechanism by which the MPCs differentiate into presomitic mesoderm (PSM) cells remains largely unclear. Especially, the molecules that promote PSM differentiation during tail development should be clarified. Here, we show that zebrafish embryos defective in *mesogenin1* (*msgn1*) and *spt* failed to differentiate into PSM cells in tail development and show increased expression of *wnt8* and *ntl*. Msgn1 acted in a cell-autonomous manner and as a transcriptional activator in PSM differentiation. The expression of *msgn1* initially overlapped with that of *ntl* in the ventral tailbud, as previously reported; and its mis-expression caused ectopic expression of *tbx24*, a PSM marker gene, only in the tailbud and posterior notochord, both of which expressed *ntl* in zebrafish embryos. Furthermore, the PSM-inducing activity of misexpressed *msgn1* was enhanced by co-expression with *ntl*. Thus, Msgn1 exercised its PSM-inducing activity in cells expressing *ntl*. Based on these results, we speculate that *msgn1* expression in association with that of *ntl* may allow the differentiation of progenitor cells to proceed during development of somites in the tail.

Introduction

In vertebrate embryos, somites gradually develop in an anterior to posterior order through the extension of the body axis. The posterior body of vertebrates is generated from progenitor cells residing in the tailbud. In zebrafish development, the tailbud is considered to contain the progenitor cells for tail neural tube, axial tissues, and somites. The progenitor cells for somites, called mesodermal progenitor cells (MPCs) and which appear to have stem cell-like characteristics, continuously produce the presomitic mesoderm (PSM) cells, which further differentiate into somites (Griffin and Kimelman, 2002). Thus, how the MPCs are maintained and how their differentiation into the PSM cells is initiated around the MPCs are key issues for understanding of the early process of somite development.

Accumulating evidence has revealed the molecular mechanism underlying the maintenance of the MPCs. Genetic studies have indicated that *Wnt* and *Brachyury* are required for the development of most of the posterior paraxial mesoderm cells (Lekven et al., 2001; Martin and Kimelman, 2008; Schultemerker et al., 1994; Takada et al., 1994; Yamaguchi et al., 1999). In zebrafish embryos, *wnt* and zebrafish orthologues of brachyury, *ntl* and *bra*, mutually activate their expressions in the MPCs; and this autoregulatory loop is essential for maintenance of the undifferentiated state of the MPC (Martin and Kimelman, 2008). In addition, *ntl* also functions for the maintenance of the MPCs by activating *cyp26a1* expression, which leads to the clearance of retinoic acid, an inhibitor of the Wnt/Brachyury autoregulatory loop (Martin and Kimelman, 2010). Bmp signaling, which is known to inhibit the expression of Wnt antagonists in the tailbud, also plays a role in the maintenance of the MPCs (Row and Kimelman, 2009). Thus, the undifferentiated state of the MPC appears to be controlled by the Wnt/Brachyury autoregulatory loop and the molecules regulating this loop as well.

On the other hand, the molecular mechanism promoting MPC differentiation into PSM cells should also be elucidated for a better understanding of the MPC-based development of the paraxial mesoderm. Of note, genetic studies with the zebrafish have indicated that the molecular mechanisms underlying the development of somites are not the same between trunk and tail. For instance, mutant embryos defective in the function of 2 nodal ligands, *cyclops* and *squint*, or in that of a component of the nodal receptor complex, *one-eyed pinhead* (*oep*), completely lack the trunk mesoderm including somites (Feldman et al.,1998; Gritsman et al.,1999), but develop

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relatively normally tail somites. In addition, *spadetail* (*spt*)/*tbx16* mutant embryos are impaired in the development of their trunk somites, but generate relatively normal tail ones (Griffin et al., 1998). These findings suggest that there is some difference between trunk and tail somite development in terms of the machinery that regulates MPC differentiation into PSM cells. Interestingly, *spt* mutant embryos show impaired differentiation of MPCs into PSM cells during the development of the trunk, but not during that of the tail (Griffin et al., 1998). However, in contrast to this phenotype restricted to the trunk somites, other evidence suggests that *spt* is required for PSM differentiation in the development of tail somites, as well. For instance, *spt* mutant embryos treated with a low dosage of a chemical inhibitor of the FGF receptor lack their entire skeletal muscles including tail muscles (Griffin and Kimelman, 2003). Furthermore, by additional depletion of zygotic *one-eyed pinhead* (*oep*) function, tail PSM differentiation is arrested in *spt* mutant embryos (Griffin and Kimelman, 2002). These findings indicate that *spt* is required for PSM differentiation in both trunk and tail somites and that some additional factors compensate for the loss of Spt function during tail development. Therefore, for understanding the molecular mechanism that controls the maintenance and subsequent differentiation of the MPCs, it is important to reveal the function of Spt and these additional factors during the development of tail somites, especially in terms of their interaction with the Wnt/Brachyury autoregulatory loop.

One candidate as an additional factor seems to be Mesogenin1 (Msgn1), which is a bHLH transcription factor expressed in the PSM (Joseph and Cassetta, 1999; Yoo et al., 2003; Yoon et al., 2000). Interestingly, mouse embryos deficient in functional Msgn1 have impaired development of their posterior somites, in spite of having normal formation of the first 7 somites, as well as show an abnormal accumulation of an undifferentiated cell mass at the tip of their tail (Yoon and Wold, 2000). Thus, Msgn1 seems to be involved in PSM differentiation during the development of posterior, or tail, somites. However, it is still uncertain as to how the differentiation from the MPCs to PSM cells is controlled by *msgn1* during somite development. Furthermore, it has remained to be elucidated whether zebrafish *msgn1* interacts with *spt* during PSM differentiation during tail development. In this study, we assessed the functions of *msgn1* in zebrafish development by injecting *msgn1* specific MO into wild-type and *spt* mutant eggs. Our results show that both *msgn1* and *spt* were required for PSM differentiation from the MPCs during tail development. This result and additional evidence uncovered the mechanism underlying the differentiation of MPCs into PSM cells, one in which *msgn1* and *spt* play key roles, during tail development.

Experimental procedures

Fish and embryos

Zebrafish with the TL2 background were used as described previously (Kishimoto et al., 2004). For generation of *spt* mutant embryos, the *spt*^{kt378b} mutant strain, which was obtained by an ENU-based mutant screening in our laboratory (Koshida et al., 2005), was also used. This *spt*^{kt378b} strain carries a point mutation in the splicing acceptor of the 1st intron (Supplementary Fig. S1). Collected embryos were grown at 28.5 °C or 23.5 °C, and their developmental stages were determined according to morphological criteria.

Genotyping of spt^{kt378b} *mutant embryos*

For the genotyping of *spt*^{kt378b} mutant embryos, genomic DNA fragments amplified by PCR using spt-cr1(TTTCTGAAAACAAAACACACAACA) and spt-gt2(GCTAAATAATGCAGGCTATCCGAG) were digested with BsaJI, which can digest the DNA fragment from the wild type but not that from *spt*^{kt378b}.

Morpholino oligonucleotides

The sequences of the MOs used were the following: CACATCCACGTCGATTTGCGCCATG for *msgn1*; CACATCCACgTCgATTTgCgCCATg for 5mis *msgn1*; and GCTTGAGGTCTGATAGCCTGCAT for *spt*.

Plasmid construction

To generate capped *msgn1* RNA, we amplified the open reading frame of zebrafish *msgn1* by RT-PCR and cloned it into the EcoRI and XbaI sites of pCS2+ vector for *in vitro* transcription. For synthesis of RNA probes, the full-length *msgn1* cDNA containing the 3′UTR was amplified by PCR with the primers shown below and cloned into BamHI and KpnI sites of pBS-SK+. To generate the expression vector containing the *msgn1* promoter, we utilized the tol2 vector system with some modifications. DNA fragments containing the left and right arms of tol2 were cut out from pT2AUASMCS (Kawakami Lab.) by using SacI-NotI and ApaI-KpnI and cloned into the SacI-NotI and ApaI-KpnI sites, respectively, of pBS SK+. Next we cut out the fragment containing the SV40 polyA adenylation sequence from pCS2SN by using ApaI-NotI and subcloned it into the ApaI-NotI site of the pBS-tol2A vector (pSK-tol2B). The *msgn1* promoter was isolated by cutting out the2967 kbps fragment from the ScaI and Kpn21I from a bac clone (DKEY-66N8) and cloned into the pSK-tol2B vector (sk+tol2 *msgn1*). The 2A peptide sequence was added to the C-terminus of mCherry by PCR. Then the *msgn1* ORF without its start codon was amplified by PCR using GGAATTCAGCGCAAATCGACGTGGATGT and GGTCTAGAATCACTGCTGCTCGAGGATGC, and connected to the 3′ of mCherry-2a fusion cDNA and cloned into the sk+tol2 *msgn1*. ΔN-*msgn1* cDNA was amplified by PCR using GGAATTCAAAAGTGAAGATGAGTATGAGGAG and GGTCTAGAATCACTGCTGCTCGAGGATGC. To obtain Δbasic cDNA, we generated N-terminal and C-terminal fragments of Msgn1 by PCR using the following respective primers: GGAATTCGCGCAAATCGACGTGGATGT/CCTCCGCCAGACTCCTCATCATACTCATCTTCACTTTCGG and CCGAAAGTGAAGATGAGTATGATGAGGAGTCTGGCGGAGG/GGTCTAGAATCACTGCTGCTCGAGGATGC. Then these fragments were connected by PCR. For the construction of VP16- and EnR-msgn1, the C-terminal domain of *msgn1* was amplified by PCR using GGAATTCAAAAGTGAAGATGAGTATGAGGAG and GGTCTAGAATCACTGCTGCTCGAGGATGC and cloned into pCS2+NLSVP16AD and pCS2+EnR, respectively. These mutant forms of *msgn1* were connected to the 3′ region of mCherry-2a fusion cDNA and cloned into sk+tol2 *msgn1*.

In situ hybridization

In situ hybridization was performed as described previously (Jowett and Yan, 1996; Jülich et al., 2005). In experiments where the signals were detected with fluorescence, anti-FITC-HRP (Invitrogen) was used. Probes to detect the following mRNAs were used, *msgn1*, *papc* (Yamamoto et al., 1998), *tbx24* (Nikaido et al., 2002), *ntl* (Schultemerker et al., 1994), *wnt8* (Kelly et al., 1995), *floating head* (*flh*) (Talbot et al., 1995), *sox2*, and *pax2a* (Krauss et al., 1992).

Transplantation

The transplantation experiment was performed as described previously (Kawamura et al., 2005). Cells isolated from host embryos that had been injected with 1% rhodamine, 2.5 ng *spt* MO (GCTTGAGGTCTCTGATAGCCTGCAT), and 2 ng *msgn1* MO