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Maternal Vsx1 plays an essential role in regulating prechordal mesendoderm and forebrain formation in zebrafish

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ABSTRACT

Prechordal mesendoderm (PME) is a derivative of gastrula organizer underlying the anterior neural plate of vertebrate embryos. It has been firmly established that PME is critical for head induction and anterior–posterior patterning. Therefore, the establishment of PME in a desired shape and size at a correct position during early embryogenesis is crucial for normal head patterning. However, it remains largely unclear how the desired form and size of PME is generated at a predestined position during early embryogenesis. Here we show that in zebrafish a maternal transcription repressor Vsx1 is essential for this early developmental regulation. Knocking down maternal *vsx1* resulted in impaired PME formation and progression associated with a deficient and posteriorized forebrain. Loss- and gain-of-function experiments showed that maternal Vsx1 is essential for repressing *ntl* ectopic expression in more animal region at early gastrula stages. Chromatin immunoprecipitation assay in combination with core consensus sequence mutation analysis further revealed that maternal Vsx1 can directly repress *ntl* transcription by binding to the proximal promoter at a specific site. Simultaneous inhibition of *ntl* function could successfully suppress the defects of both PME and forebrain formation in maternal Vsx1 knockdown embryos. Our results reveal a pivotal role for maternal Vsx1 as a direct transcriptional repressor of *ntl* expression at the margin of the zebrafish gastrula to ensure directional cell polarization and migration of PME cells.

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Introduction

Prechordal mesendoderm (PME) is a derivative of gastrula organizer underlying the anterior neural plate of vertebrate embryos. It has been firmly established that in vertebrates PME is critical for forebrain induction and anterior–posterior patterning during gastrulation (reviewed by Kiecker and Niehrs, 2001; Wilson and Houart, 2004; Andoniadou and Martinez-Barbera, 2013). Therefore, the establishment of PME in a desired shape and size at a correct position is critical for normal head patterning. As a derivative of the gastrula organizer, the formation of PME is initiated in response to the activity of maternal β-catenin at the prospective dorsal region in both *Xenopus* (Harland and Gerhart, 1997) and zebrafish (Kelly et al., 2000). In zebrafish, graded nodal activities are involved in establishing the anteroposterior polarity of the pregastrula organizer. Nodal gain- and loss-of-function studies reveal that high level of nodal signaling is essential for specifying the PME cells at the anterior region of organizer (Gritsman et al., 2000; Thisse et al., 2000; Dougan et al., 2003). PME cells involute at the beginning of gastrulation and then migrate anteriorly until reaching a given position beneath the neural plate during gastrulation (Kiecker and Niehrs, 2001). A number of transcription factors, including Goosecoide (Gsc) (Ferreiro et al., 1998; Latinkic and Smith, 1999; Yao and Kessler, 2001) and Otx2 (Pannese et al., 1995; Acampora et al., 1995; Foucher et al., 2006), are expressed in the presumptive PME cells to promote PME activity. The embryos lacking the function of either Gsc or Otx2 exhibit the failure of PME formation associated with deletion of forebrain (Pannese et al., 1995; Latinkic and Smith, 1999; Yao and Kessler, 2001).

In *Xenopus*, simultaneous inhibition of Bmp and Wnt signaling is necessary for head induction (Glinka et al., 1997). PME cells secret several Wnt and Bmp antagonists, such as Dkk1 and Cerberus, to protect forebrain formation. Wnt antagonist Dkk1 is expressed within the PME at late gastrula stage in zebrafish (Hashimoto et al., 2000; Shinya et al., 2000), *Xenopus* (Glinka et al., 1998; Kazanskaya et al., 2000; Niehrs et al., 2001) and mice (Mukhopadhyay et al., 2001). Loss of Dkk1 function results in microcephaly or absence of anterior structure, whereas overexpression of Dkk1 leads to enlargement of the head (Caneparo et al., 2007). Cerberus is a secreted inhibitor of nodal, Bmp and Wnt

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signals in the extra-cellular space to protect head formation (Piccolo et al., 1999). Misexpression of cerberus can induce ectopic heads (Bouwmeester et al., 1996). Therefore, normal PME formation at a correct position of the embryo is critical for normal forebrain formation and location. However, it remains largely unclear how the PME is generated in a desired form and size at a given position during early embryogenesis.

Visual system homeobox-1 (*vsx1*) is a paired-like transcription factor gene (which encodes a protein containing a homeodomain and a CVC domain) originally cloned from an adult goldfish retina cDNA library (Levine and Schechter, 1993). So far, homologs of Vsx1 have been identified in several vertebrate species, including zebrafish (Passini et al., 1998), *Xenopus* (D'Autilia et al., 2006), chick (Chen and Cepko, 2000), mouse (Ohtoshi et al., 2001) and human (Semina et al., 2000). In all the examined vertebrate species, *vsx1* is expressed during early developmental stages and in the adult retina (Levine et al., 1994; Passini et al., 1998; Semina et al., 2000; Ohtoshi et al., 2001; D'Autilia et al., 2006). Previous studies have identified that Vsx1 participates in regulating retinal progenitor proliferation, differentiation and functional maintenance of bipolar cells (Hayashi et al., 2000; Héon et al., 2002; Chow et al., 2004; Ohtoshi et al., 2004; Valleix et al., 2006; Clark et al., 2008) and proposed that Vsx1 might also play an important role during early embryogenesis (Ohtoshi et al., 2001). Recently, it has been observed that, by directly repressing the transcription of a key axial mesoderm regulatory gene in ventrolateral mesoderm region, maternal Vsx1 plays a pivotal role in axial–paraxial mesoderm patterning during early embryogenesis in zebrafish (He et al., 2014). In maternal *vsx1* knockdown embryos, the axial mesoderm domain is shorter than that in the wild type embryos at bud stage (He et al., 2014), implying that maternal Vsx1 is involved in regulating anterior–posterior patterning in zebrafish. Here we show that maternal Vsx1 plays an essential role in PME formation and progression, as well as normal forebrain formation during early embryogenesis in zebrafish.

Results

Maternal Vsx1 is essential for normal PME formation and progression

To determine the role of maternal Vsx1 in anterior–posterior patterning, we examined PME formation and position in maternal Vsx1 knockdown embryos at bud stage. Maternal Vsx1 knockdown was performed by injecting 3.8 ng of a translation blocking morpholino oligonucleotides (tbMO), which targets to the ATG region of *vsx1* mRNA specifically as described previously (He et al., 2014). Spatial pattern of anterior mesendoderm, chordamesoderm, neural plate and epidermis were visualized in wild type and *vsx1* tbMO injected embryos by whole-mount in situ hybridization with anterior mesendoderm marker *hgg1*, axial mesoderm marker *ntl*, the neural and non-neural ectoderm boundary marker *dlx3b*. In about 65% of the *vsx1* tbMO injected embryos ($N=43$), *hgg1* marked PME domain was strikingly smaller than that in the wild type embryos, while *ntl* marked axial mesoderm domain was expanded in width but shortened in length, and the neuroectoderm domain was much broader in comparison with that in the wild type control (Fig. 1A–H). Judging by the position of leading edge of the neuroectoderm, the whole *hgg1* marked PME domain in the wild type was located at the anterior side (Fig. 1B and C), but in *vsx1* morphants was located at the posterior side (Fig. 1F and G). The distance between the posterior edge of the *hgg1* domain and the anterior edge of *ntl* domain was much shorter than that in the wild type embryos (Fig. 1B and F). The defects of PME formation and position in maternal Vsx1 knockdown embryos indicate that maternal Vsx1 is essential for PME formation and progression during early embryogenesis.

Whole-mount in situ hybridization with differentially labeled chordamesoderm marker *ntl* and PME maker *gsc* showed that *gsc* marked PME cells were converged to the dorsal midline, separated from the *ntl* marked margin and migrating toward the animal pole at 40% epiboly stage in the wild type control (Fig. 2A and B). In the *vsx1* tbMO injected embryos, however, the *gsc* marked PME cells were not converged to the dorsal midline and detained within the animal-expanded margin marked by *ntl* (Fig. 2D and E). It appears that the presumptive PME cells in *vsx1* tbMO injected embryos are unable to migrate toward the animal pole.

Elongation of the cells at the leading edge of the mesendoderm, close to the border between epiblast and hypoblast, is a morphological feature previously reported to correlate with the migratory activity of prechordal plate cells at the onset of zebrafish gastrulation (Montero et al., 2003; Ulrich et al., 2003). To determine whether the presumptive PME cells in the morphants are migratory, we analyzed changes in cell shape of anterior mesendoderm cells during gastrula stage. Cell shape of the embryos was displayed by membrane-bound YFP. At 60% epiboly stage, cells at the leading edge of the mesendoderm, close to the border between epiblast and hypoblast, were elongated and migrated toward the animal pole in the wild type embryos (Fig. 2C and C′) but not in the tbMO injected siblings (Fig. 2F and F′). These results demonstrated that maternal Vsx1 regulates PME formation by protecting the directional elongation and migration of presumptive PME cells.

Maternal Vsx1 indirectly regulates forebrain formation and position

We also observed remarkable defects of head formation in the *vsx1* tbMO injected embryos at 24 hours post fertilization (hpf). When 3.8 ng *vsx1* tbMO was injected at one cell stage, about 60% of injected embryos ($N=152$) exhibited dorsalized phenotype with dramatically reduced brain at 24 hpf, in which no distinct brain ventricles could be observed (Fig. 3C–D′). When coinjected with 230 pg *vsx1* mis-mRNA (encoding the normal Vsx1 protein but lacking the target site of the *vsx1* tbMO), brain ventricles were detectable in about 70% of embryos ($N=155$, Fig. 3I), suggesting that the brain defects in *vsx1* tbMO injected embryos were due to a reduction of endogenous Vsx1 protein level. Previously it was shown that zygotic *vsx1* mRNA was synthesized at the 14-somite stage (16 hpf) in the central nervous system (Passini et al., 1998). To determine whether maternal *vsx1* mRNA alone or both maternal and newly synthesized zygotic *vsx1* mRNA are involved in regulating brain patterning, we used a splice-blocking MO (sbMO). The sbMO targets to the first splice site of *vsx1* mRNA and has been demonstrated to block the splicing of newly synthesized zygotic *vsx1* mRNA specifically and effectively (Clark et al., 2008) but leave the maternal deposited *vsx1* mRNA intact. However, injection with 3.8 ng to 15 ng of sbMO elicited no detectable brain defect until 24 hpf (Fig. 3E–F′), though eye abnormality was detected at late developmental stages as observed in previous experiments (Clark et al., 2008; He et al., 2014). Hence, it is maternal Vsx1, rather than zygotic Vsx1, that is essential for brain patterning.

Brian defects caused by loss of maternal Vsx1 function were analyzed in detail by whole-mount in situ hybridization with forebrain, midbrain and hindbrain markers at 8–10 somite stage. In comparison with the wild type control, the expression of forebrain marker gene *six3b* in *vsx1* tbMO injected embryo was significantly repressed and its expression domain was smaller and located at a posterior position ($N=30/36$, Fig. 4A–B′). However, no detectable repression of both midbrain marker gene *eng2a* and hindbrain marker gene *krox20* was observed in the examined *vsx1* tbMO injected embryos ($N=37/37$ and 29/29, respectively),

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