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An essential and highly conserved role for Zic3 in left–right patterning, gastrulation and convergent extension morphogenesis

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ABSTRACT

Mutations in ZIC3 result in X-linked heterotaxy in humans, a syndrome consisting of left–right (L–R) patterning defects, midline abnormalities, and cardiac malformations. Similarly, loss of function of Zic3 in mouse results in abnormal L–R patterning and cardiac development. However, *Zic3* null mice also exhibit defects in gastrulation, neural tube closure, and axial patterning, suggesting the hypothesis that Zic3 is necessary for proper convergent extension (C-E) morphogenesis. To further investigate the role of Zic3 in early embryonic development, we utilized two model systems, *Xenopus laevis* and zebrafish, and performed loss of function analysis using antisense morpholino-mediated gene knockdown. Both *Xenopus* and zebrafish demonstrated significant impairment of C-E in Zic3 morphants. L–R patterning was also disrupted, indicating that the role of Zic3 in L–R axis development is conserved across species. Correlation of L–R patterning and C-E defects in *Xenopus* suggests that early C-E defects may underlie L–R patterning defects at later stages, since Zic3 morphants with moderate to severe C-E defects exhibited an increase in laterality defects. Taken together, these results demonstrate a functional conservation of Zic3 in L–R patterning and uncover a previously unrecognized role for Zic3 in C-E morphogenesis during early vertebrate development.

Introduction

ZIC3 is a zinc finger transcription factor of the GLI superfamily. Loss of function mutations in ZIC3 result in X-linked heterotaxy, a syndrome consisting of left–right (L–R) patterning defects of the visceral organs, midline abnormalities and cardiac malformations (Gebbia et al., 1997; Ware et al., 2004). There are five *Zic* genes (*Zic1-5*) in human, mouse and *Xenopus laevis* (Aruga et al., 1994, 1996; Brown et al., 1998; Fujimi et al., 2006; Gebbia et al., 1997; Yokota et al., 1996). Seven *zic* genes (*zic1*, *zic2a*, *zic2b*, *zic3*, *zic4*, *zic5* and *zic6*) have been described in zebrafish (Merzdorf, 2007). Across species, Zic proteins are highly conserved within their DNA-binding domains, which consist of five C_2H_2 zinc-finger motifs (Aruga, 2004; Herman and El-Hodiri, 2002; Keller and Chitnis, 2007). Despite the high conservation and close evolutionary relationship of the *Zic* gene family, only *Zic3* has been shown to be required for proper L–R asymmetry.

L–R patterning depends on a conserved pathway that includes asymmetric TGFβ signaling on the left side of the embryo (Shiratori and Hamada, 2006; Whitman and Mercola, 2001). Nodal, a TGFβ ligand, activates asymmetric expression of the transcription factor *Pitx2* which is thought to mediate L–R morphogenesis of developing organs. *Zic3* null mice exhibit L–R asymmetry defects recapitulating human heterotaxy syndrome (Purandare et al., 2002; Ware et al., 2006b). *Zic3* null embryos fail to maintain *Nodal* expression, and Zic3 has been shown to activate a Nodal enhancer in *Xenopus* and mouse (Ware et al., 2006a). Later in development, *Zic3* null mice exhibit randomization of *Nodal* and *Pitx2* in the lateral plate mesoderm, as well as abnormalities of asymmetric organs including the heart, lung, liver and spleen (Purandare et al., 2002). These findings emphasize a role for Zic3 upstream of Nodal signaling in specifying L–R asymmetry.

Previous studies have also implicated Zic3 in early embryo patterning and differentiation (Lim et al., 2007, 2010; Ware et al., 2006b). Loss of *Zic3* in embryonic stem cells leads to loss of pluripotency and ultimately endoderm differentiation (Lim et al., 2007). *In vivo*, *Zic3* null mice exhibit abnormalities of anterior visceral endoderm patterning (Lim et al., 2007; Ware et al., 2006b). Consistent with a role in early development, *Zic3* is highly expressed in neuroectoderm and mesoderm during gastrulation of mouse, chick, *Xenopus* and zebrafish embryos (Keller and Chitnis, 2007; Kitaguchi et al., 2000; McMahon and Merzdorf, 2010; Nagai et al., 1997; Nakata et al., 1997). This conserved expression pattern suggests a potential role for Zic3 during gastrulation and axial patterning.

Zic3 null mice exhibit defective gastrulation, neural tube closure and axial patterning (Purandare et al., 2002; Ware et al., 2006b)

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indicating important roles for this transcription factor during early embryogenesis. Elongation of the anterior–posterior (A–P) axis and closure of the neural tube are dependent on the morphogenetic process convergent extension (C-E), which is regulated by one of the non-canonical Wnt pathways, also known as the planar cell polarity (PCP) pathway (Keller et al., 1985; Wallingford and Harland, 2002). This dynamic process requires a tight balance of cell adhesion and interaction in order for cells in a tissue layer to act as one. C-E morphogenesis defects have been well characterized in *Xenopus* embryos, which include blastopore closure defects during gastrulation, flexion and shortening of the A–P axis, and impaired neural tube elongation and closure (Sokol, 1996; Sumanas and Ekker, 2001; Wallingford and Harland, 2001; Wallingford and Harland, 2002). Similarly, C-E defects in mouse include a specific subtype of neural tube defect, shorter and broader body axis, as well as failure of somitic tissue to converge at the midline (Garcia-Garcia et al., 2008; Lu et al., 2004; Wang et al., 2006; Yen et al., 2009). In zebrafish, C-E defects manifest as widening of the neural plate and somites, as well as a reduction in A–P axis length (Henry et al., 2000; Topczewski et al., 2001). *Zic3* null mice exhibit thickening of the primitive streak and failure of the notochord to delaminate from the foregut tissue, which suggests defective C-E since these tissues are not able to converge properly at the midline (Purandare et al., 2002; Ware et al., 2006b).

To gain insight into the role of Zic3 during early embryo development, a loss of function approach was taken using *X. laevis* and zebrafish as model vertebrates. This study addressed 1) the role of Zic3 during C-E morphogenesis, 2) conservation of Zic3 function during L–R patterning and 3) whether C-E defects lead to L–R patterning defects. Results from this study show that Zic3 has a conserved role in *Xenopus* and zebrafish that is essential for normal gastrulation, C-E morphogenesis and L–R patterning. In addition, this study suggests that proper C-E is a prerequisite for normal L–R axis formation in vertebrates.

Materials and methods

Embryo collection and staging

X. laevis embryos were obtained through *in vitro* fertilization. Adult females were primed with 800–1000 U of human chorionic gonadotropin (Chorulon). Testes were isolated from adult males and stored in oocyte culture medium (OCM) at 4 °C. Embryos were dejellied in 2% cysteine (Sigma, pH 7.8) and cultured in 0.01× Marc's Modified Ringer (MMR). Embryo staging was determined according to the *X. laevis* developmental table (Nieuwkoop and Faber, 1994). Wild type AB zebrafish were purchased from the Zebrafish International Resource Center (ZIRC). Zebrafish maintenance, mating, embryo culture (Westerfield, 1995) and embryo staging (Kimmel et al., 1995) were performed as previously described.

Preparation of Zic3 RNA

RNA was isolated from stage 19 *X. laevis* embryos using Trizol (Invitrogen) according to the manufacturer's protocol. A full length 1.4 kb cDNA was amplified by RT-PCR, sequenced in its entirety, and subsequently subcloned into pCS2+. RNA was generated using the mMessage mMachine kit according to the manufacturer's protocol (Ambion). Human ZIC3 RNA was generated similarly using a previously described HA-ZIC3 construct as template (Ware et al., 2004).

In vitro transcription/translation

The TnT coupled reticulocyte lysate system (Promega) was used for *in vitro* transcription and translation in the presence of ^{35}S-Met as per the manufacturer's instructions. *Xenopus zic3* and human *ZIC3* with an HA epitope tag were used as templates, with or without morpholino.

Zic3 antisense morpholino oligonucleotide design

For *X. laevis*, two Zic3 antisense morpholino oligonucleotides, a translational-blocking and splice site morpholino (TB MO and SS MO, respectively), were designed (Gene Tools, LLC). The TB MO sequence was 5′ ATG ACA ATG CTA TTA GAT GGA GGA C 3′, and the SS MO, designed to the exon 2–intron 2 junction, sequence was 5′ AGC ACA TGA AGG TAA GTT TTA TTG T 3′. Both Zic3 morpholinos were fluoroscein conjugated. A control antisense morpholino was also obtained from Gene Tools, LLC with the following sequence 5′ CCT CTT ACC TCA GTT ACA ATT TAT A 3′. For Zic3 knockdown in zebrafish, a Zic3 TB MO was purchased from Gene Tools, LLC with the following sequence: 5′ TAT CAA GGA GCA TAG TCA TTG GGC T 3′.All morpholinos were resuspended and stored according to manufacturer's protocol (Gene Tools, LLC).

RT-PCR analysis of Zic3 MO efficiency

In *Xenopus*, the efficiency of Zic3 knockdown by the SS MO was analyzed via RT-PCR using *zic3* full length primers: sense 5′ CTG CTC AGC TCA TTC ATG 3′, antisense 5′ GGC CTC TCT ACA TTT TGC TC 3′ and primers designed to flank exon 2: sense 5′ TCA TAT CAG GGT GCA TAC CG 3′, antisense 5′ TCC TCA CTG TTG GCA GAA ACC A 3′. ODC (*ornithine decarboxylase*) primers were used to verify RNA quantity and quality: sense 5′ GCC ATT GTG AAG ACT CTC TCC ATT C 3′, antisense 5′ TTC GGG TGA TTC CTT GCC AC 3′ (Heasman et al., 2000).

Embryo microinjection

Xenopus microinjections were performed in 4% Ficoll in 1/3× MMR and were targeted to the dorsal marginal zone of both dorsal blastomeres of 4-cell stage embryos. *Xenopus* embryos were injected with 5.3 ng Zic3 MO. Zebrafish embryos were injected with 7.5 ng Zic3 MO at the 1–2 cell stages. *In vitro* transcribed *Zic3* mRNA was injected into zebrafish Zic3 morphants in order to rescue heart looping (125 pg) and body axis (50 pg) phenotypes.

Whole-mount in situ hybridization (WISH)

Xenopus embryos were fixed at the desired stage in MEMFA for 2 h at room temperature. Following fixation, embryos were dehydrated in 100% EtOH and stored at −20 °C. WISH was performed as previously described (Sive et al., 2000). pMyoD, pSP73-Xbra, pBSK-Cardiac Troponin, and pCS107-Pitx2 plasmids were kindly provided by the Zorn laboratory and were used to generate antisense riboprobes. Zebrafish embryos were fixed at the stages indicated and processed following the procedure previously reported (Essner et al., 2005). Antisense probes for *myoD*, *dlx3*, *southpaw* (*spaw*), *cardiac myosin light chain 2* (*cmlc2*), *forkhead box a3* (*foxa3*), and *no tail* (*ntl*) were generated as described (Essner et al., 2005). Images were captured using a Zeiss MRc AxioCam digital camera mounted on a Zeiss Discovery V12 stereomicroscope. Measurements were made using AxioVision software (Zeiss).

Anatomic and morphological analysis of embryos

Xenopus embryos were anesthetized in 0.1% benzocaine (Spectrum Chemical Company) in 0.1× MMR at stage 46 to analyze heart and gut anatomy. Once anesthetized, *Xenopus* heart morphology was analyzed and scored immediately utilizing blood flow to determine the positioning of the outflow tract (Branford et al., 2000). After fixation, WISH was performed using the cardiac marker *troponin* to mark the heart. The anatomic characterization of laterality