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Wnt/β-catenin signaling controls *Mespo* expression to regulate segmentation during *Xenopus* somitogenesis

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Abstract

The vertebral column is derived from somites, which are transient segments of the paraxial mesoderm that are present in developing vertebrates. The strict spatial and temporal regulation of somitogenesis is of crucial developmental importance. Signals such as Wnt and FGF play roles in somitogenesis, but details regarding how Wnt signaling functions in this process remain unclear. In this study, we report that Wnt/β-catenin signaling regulates the expression of *Mespo*, a basic–helix–loop–helix (bHLH) gene critical for segmental patterning in *Xenopus* somitogenesis. Transgenic analysis of the *Mespo* promoter identifies *Mespo* as a direct downstream target of Wnt/β-catenin signaling pathway. We also demonstrate that activity of Wnt/β-catenin signaling in somitogenesis can be enhanced by the PI3-K/AKT pathway. Our results illustrate that Wnt/β-catenin signaling in conjunction with PI3-K/AKT pathway plays a key role in controlling development of the paraxial mesoderm.

Keywords: AKT; Paraxial mesoderm; PI3-K; Wnt; *Xenopus laevis*

Introduction

During vertebrate embryogenesis, the paraxial mesoderm separates into somites, a metameric series of homologous subunits, which bud off sequentially from the rostral end of the presomitic mesoderm (PSM) at regular intervals along the anterior–posterior (AP) axis. The process in which the segmental or metameric pattern is established accompanying the constant posterior elongation of the embryo body axis is termed somitogenesis (Weisblat et al., 1994). Somitogenesis occurs in a strict spatial and temporal progression (Dubrulle et al., 2001; Pourquie, 2003).

The molecular nature of the signals responsible for controlling somitogenesis remains elusive. One potential candidate for regulating this process is Wnt/β-catenin signaling (Aulehla et al., 2003; Yamaguchi et al., 1999). Previous studies have shown that Wnt/β-catenin signaling is implicated in controlling the development of paraxial mesoderm (Aulehla et al., 2003; Galceran et al., 2004; Hofmann et al., 2004; Pourquie, 2001). In mouse embryos, *Wnt3a* is expressed in the primitive streak during gastrulation and in the tailbud during the later stages of mouse development. Animals homozygous for null alleles of *Wnt3a* lack somites posterior to the forelimbs (Ikeya and Takada, 2001; Takada et al., 1994; Wilson et al., 1993). The double mutant harboring mutations in *lef1* and *tcf1*, identified as nuclear mediators of Wnt/β-catenin signaling that activate Wnt-responsive genes by associating with β-catenin, results in lack of paraxial mesoderm resembling the *Wnt3a* null mutation (Galceran et al., 1999). In chick embryos, it has been shown that altering Wnt/β-catenin signaling activity in the PSM causes defects in somite formation (Aulehla et al., 2003). However, the exact molecular nature of Wnt/β-catenin signaling in somitogenesis is not fully understood, and whether Wnt/β-catenin signaling is involved in somitogenesis of the lower vertebrates such as *Xenopus* is still questionable.

The FGF signaling pathway also participates in somitogenesis (Delfini et al., 2005; Dubrulle et al., 2001; Moreno and Kintner, 2004). *Fgf8* mRNA forms a gradient along the PSM in mouse

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embryos (Delfini et al., 2005; Dubrulle et al., 2001). Altering the activity of FGF signaling in the PSM resulted in segmental defects (Dubrulle et al., 2001). These data demonstrate that FGF signaling is required for somitogenesis. It has been proposed that FGF signaling may act through the PI3-K/AKT pathway in the PSM to participate in somitogenesis (Dubrulle and Pourquie, 2004). Since PI3-K/AKT signaling can enhance accumulation of β-catenin by inhibiting GSK-3β during cardiomyocyte growth (Haq et al., 2003), it may regulate Wnt/β-catenin activity in the segmentation process by a similar mechanism.

Somite formation and patterning are thought to be controlled by *Mespo*, a member of the basic–helix–loop–helix (bHLH) transcription factor family (Moreno and Kintner, 2004; Yoon et al., 2000; Yoon and Wold, 2000). *Mespo* expression encompasses the entire caudal PSM of *Xenopus* embryos, and its expression is reduced when the PSM cells enter the segmentation process (Joseph and Cassetta, 1999; Kim et al., 2000; Moreno and Kintner, 2004). Gain of function experiments showed that *Mespo* can induce expression of paraxial mesoderm marker genes (Yoon et al., 2000). *Mespo* null mouse embryos exhibited no identifiable somites or segmental patterning in the trunk posterior to forelimbs, and severe disruption of gene expressions in posterior paraxial mesoderm (Yoon and Wold, 2000). These results suggest that *Mespo* plays essential roles in somitogenesis. The phenotypic resemblance between *Mespo* and *Wnt3a* mutant mouse embryos provides a strong hint that Wnt/β-catenin signaling regulates the expression of *Mespo* during paraxial mesoderm development. However, whether *Mespo* expression is regulated by Wnt/β-catenin signaling in the PSM remains unclear.

Here, we report that Wnt/β-catenin signaling directly regulates the expression of *Mespo*, and this regulation is enhanced by PI3-K/AKT signaling. These results demonstrate that Wnt/β-catenin signaling plays a key role in controlling somitogenesis by regulating *Mespo* gene expression, and that PI3-K/AKT signaling is also involved in this process.

Materials and methods

Embryo manipulations, drug treatment

Preparation and injection of *Xenopus* embryos were carried out as previously described (Ding et al., 1998). Embryos were staged according to Nieuwkoop and Faber (1967). LY294002 (Calbiochem) was used at 100 μM. Chemicals were dissolved in DMSO and then diluted into 0.1× MMR. In each case, about 20 embryos were examined per condition per probe. Each experiment was repeated three times independently.

Morpholino oligomer and in vitro translation

The 25-bp morpholino antisense oligomer for *Xenopus Mespo* was obtained from Gene Tools and consisted of the following sequence:

*Mespo*MO: GGGATGGTGCAGAGTCTCCATCAGT

Morpholino was dissolved in water to a concentration of 1 mM, which was then further diluted to a give a working solution of 0.3 mM. For the rescue assay, we generated a mutated *Mespo* cDNA (m*Mespo*) differed in seven bases (underlined) from *Mespo*: 5′-CTACTATGGAGACTCTGCACCATCCCCT.

The *in vitro* transcription/translation of *Mespo* was performed according to the protocol of TNT coupled reticulocyte lysate system (Promega, L4610).

In situ hybridization, immunocytochemistry

Whole-mount *in situ* hybridization was performed as previously described (Harland, 1991) using DIG-labeled probes. For bleaching of wild-type embryos, hybridized embryos were treated with bleaching solution (0.5× SSC with 1% hydrogen peroxide and 5% formamide) under a fluorescent light. The following plasmid templates were linearized, and DIG-labeled antisense RNA probes were made using either T7 or SP6 RNA polymerase: *Thylacine1* (Sparrow et al., 1998); *Mespo* (Joseph and Cassetta, 1999); *GFP* (Geng et al., 2003); *Xwnt8* (Kazanskaya et al., 2004) and *Dkk1* (Glinka et al., 1998); *Paraxis* (a PCR-amplified full-length cDNA of *Paraxis* was cloned into pCS2+); *AKT* (a PCR-amplified coding region of *Xenopus AKT* was cloned into pCS2+); *PAPC* (Kim et al., 2000); *XBra* (Smith et al., 1991).

To visualize somite size, embryos were fixed in 2% paraformaldehyde, 2% glutaraldehyde in PBS, and stained with the muscle-specific monoclonal antibody 12/101 (Kintner and Brockes, 1985). Then embryos were post-fixed in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde), embedded in paraffin, sectioned at 10 μm, and stained with DAPI.

Mutagenesis and transgenesis

The Exsite™ PCR-based site-directed mutagenesis system (Stratagene, cat. 200502-5) was used to generate the mutants of site L, p-4317mLuc and p-511m. The primers are shown below (nucleotides altered shown in bold): 5′-AGGAGACAGCAAGGTGTTAACATG-3′ (reverse) and 5′-**CTAG**ACTCC-TCCATTAAAACGCCCACT-3′ (forward).

Transgenic *Xenopus* embryos were generated as described previously (Kroll and Amaya, 1996). Plasmids used for transgenesis assays were linearized by *Not*I digestion. Three independent rounds of transgenesis were performed for each plasmid.

Western blot

For measuring phospho-Akt levels, the caudal region of *Xenopus* embryos at stage 23 were divided into three equal parts corresponding to the caudal domain, the rostral domain of the PSM and a region including the last formed somites. In these experiments, 100 embryos were used. The samples were lysed in EDTA-free RIPA (0.1% NP40, 20 mM Tris–HCl pH 8, 10% glycerol) with cocktail protease inhibitors (Bio Basics) and Na_3VO_4.

For measuring accumulation of β-catenin in the nucleus and cytosol, the caudal domain of the PSM was dissected and cultured in 1× MMR containing LY294002 at 100 μM or DMSO for 2 h. Then β-catenin in either nucleus or cytosol was extracted according to PIERCE protocol (NE-PER™ nuclear and cytoplasmic extraction reagents, 78833).

All the samples were quantitated by Bradford assay and adjusted to equal concentration before loading. Western blot was performed as described (He et al., 2005). Polyclonal antibody P14L was used to visualize β-catenin (dilution 1:2000), and anti-phospho-Akt polyclonal antibodies (dilution 1:2000; Cell Signaling Technology) were used to detect endogenous Akt and phospho-Akt. For a loading control, equal volume of proteins were separated by SDS–PAGE and visualized with Coomassie Blue.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) assay was performed, with modifications, according to the method described previously (Sachs and Shi, 2000). 100 *Xenopus* embryos were selected at stage 13 from embryos injected with appropriate plasmids, and crosslinked with 1% formaldehyde at room temperature for 20 min. Following treatment with 0.125 M glycine, embryos were washed and resuspended in 200 μl ChIP lysis buffer. The lysates were sonicated on ice to obtain DNA fragments on an average around 500 bp and immunoprecipitated with anti-HA monoclonal antibody (dilution 1:3000; Sigma). DNA fragments were washed, eluted and recovered from the immunoprecipitated complex. PCR was performed under an optimized condition: 94 °C