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Developmental Expression Pattern

Developmental expression of *Shisa-2* in *Xenopus laevis*

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ABSTRACT Shisa is an antagonist of Wnt and FGF signaling, that functions cell autonomously in the endoplasmic reticulum (ER) to inhibit the post-translational maturation of Wnt and FGF receptors. In this paper we report the isolation of a second *Xenopus* shisa gene *(Xshisa-2)*. *Xenopus* Shisa-2 shows 30.7% identity to Xshisa. RT-PCR analysis indicated that *Xshisa-2* mRNA is present throughout early development and shows an increased expression during neurula and tailbud stages. At neurula stages *Xenopus shisa-2* is initially expressed in the presomitic paraxial mesoderm and later in the developing somites. The expression profiles and pattern of *Xshisa* and *Xshisa-2* differ significantly. During gastrulation only *Xshisa* mRNA is present in the Spemann-Mangold organizer and later on becomes restricted to the neuroectoderm and the prechordal plate.

KEY WORDS: *Xenopus, shisa, presomitic mesoderm, somite*

Secreted growth factors of the Wnt and Fibroblast growth factor (FGF) families have an essential role in vertebrate development (Logan and Nusse, 2004; Böttcher and Niehrs, 2005). However, Wnt activities need to be inhibited for the correct development of the head and heart (Glinka *et al.*, 1997; Marvin *et al.*, 2001; Schneider and Mercola, 2001). The head formation promoting factors Dickkopf (Dkk) and Cerberus are secreted Wnt antagonists that regulate this signaling pathway in the extracellular space (Glinka *et al.*, 1998; Piccolo *et al.*, 1999; Mukhopadhyay *et al.*, 2001; Silva *et al.*, 2003). Shisa, a recently identified protein has been shown to inhibit Wnt as well as FGF signaling in a cell autonomous manner. It binds to the immature form of Frizzled and the FGF receptors in the ER and prevents the post-translational modifications necessary for their function (Yamamoto et. al., 2005).

FGF signaling is critical for specification of the paraxial mesoderm identity (Pourquié, 2001). In mutant mice for FGF8 and FGFR1, no paraxial mesoderm (PM) is formed (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994; Sun *et al.*, 1999). Additionally, studies in *Xenopus* have shown that eFGF is able to induce XmyoD expression in the mesoderm and specifies the myogenic cells (Fisher *et al.*, 2002). Interestingly, *fgf8* expressed in the caudal presomitic mesoderm (PSM) was recently shown to inhibit myogenesis (Dubrulle *et al.*, 2001). Wnts were also shown to be involved in the initial steps of myogenesis in mammals (Cossu and Borello, 1999). In *Xenopus* embryos, injection of *Xwnt8* RNA leads to an enlargement of the PM territory (Christian & Moon 1993), whereas injection of dominant-negative *Xwnt8* impairs the formation of the somitic territory (Hoppler *et al.,* 1996). In higher vertebrates, Wnt3a plays a critical role in maintaining the PM fate in the posterior somites (Takada *et al.,* 1994) and like Fgf8, Wnt3a gradients are also important in controlling segmentation in the PSM (Aulehla *et al.*, 2003). In this paper we report the isolation of *Xshisa*-2, an ortholog of the mouse *shisa* and describe its expression during *Xenopus laevis* embryogenesis. The expression pattern of this gene suggests that it may regulate the activities of Wnts and FGFs during *Xenopus* somitogenesis.

We have carried out a screening for differentially expressed genes in the mouse Anterior Visceral Endoderm (AVE; Filipe *et al.*, unpublished results). One of the genes identified in the screen was previously named as *mouse anterodistally expressed gene-2* (GenBank Acc. NM_145463). Through a BLAST search using this gene as query, we have identified two potential *Xenopus* homologs. One of these homologs was recently reported by Yamamoto *et. al.,* (2005) as *shisa*. The other homolog, here designated as *Xenopus shisa-2* (*Xshisa*-2), has been reported as three EST sequences (GenBank accession no. BC077953, CF286494 and BJ042155). The EST BJ042155 was obtained from NIBB (clone XL050n07; http://xenopus.nibb.ac.jp/) and the insert was completely sequenced. When compared with mouse

Abbreviations used in this paper: AVE, Anterior Visceral Endoderm; CRD, cysteine-rich domains; Dkk, Dickkopf; ER, endoplasmic reticulum; FGF, Fibroblast growth factor; FGFR, Fibroblast growth factor receptor; PM, paraxial mesoderm; PAPC, Paraxial protocadherin; PSM, presomitic mesoderm; TBD, tailbud domain; Xshisa-2, Xenopus shisa-2.

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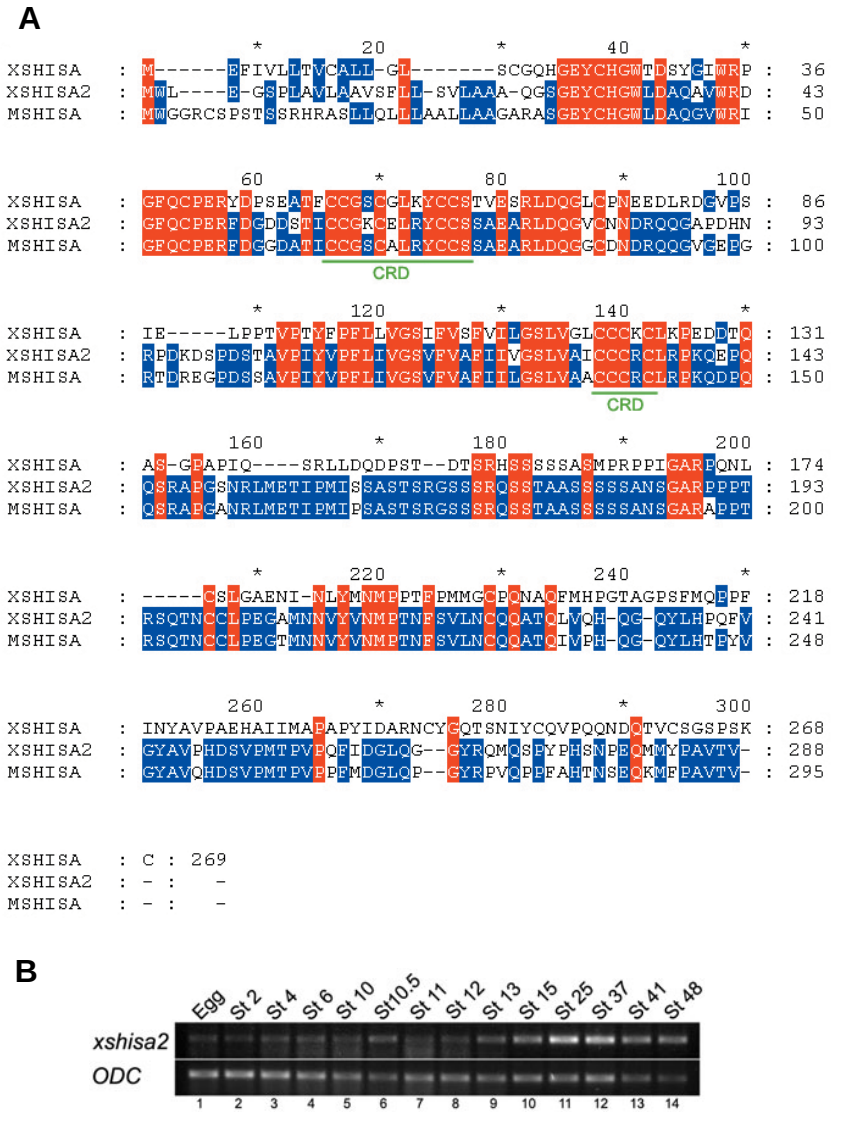

Fig. 1. Sequence alignment of Shisa family members and temporal expression of *Xshisa-2* during *Xenopus* development. (A) *Comparison of the predicted amino acid sequence of* X. laevis *Shisa-2 with* X. laevis *Shisa and mouse Shisa. Xshisa-2 shares 76.9% of identity (positives - 84.4%) with mShisa-2 and 30.7% identity (positives - 46.3%) with Xshisa. Identical amino acids among all are shaded red while identical amino acids in only two sequences are shaded blue. The absence of residues at the corresponding region is indicated by dashes. The two conserved cysteine-rich domains (CRD) are shown in green. The GenBank accession number for* X. laevis *Shisa-2 is DQ342341.* **(B)** *Temporal expression pattern of* Xenopus *Shisa-2 by RT-PCR analysis. RT-PCR was performed with total RNA from different developmental stages. Xshisa-2 transcripts are present maternally at very low levels, increase in the beginning of neurulation and continue to be expressed during early development. Stages are indicated on top. ODC was used as a loading control.*

shisa, the sequence obtained from BC077953 shows homology to the 5' of the mRNA where as BJ042155 shows homology to the 3' of the mRNA sequence. These two ESTs show partial overlapping, indicating that the ORF of Shisa-2 could be obtained from these two ESTs. When the combined sequences from these two ESTs were subjected to bioinformatic analysis, a putative open reading frame was obtained. After cloning of the full-length cDNA, we observed that this gene, *Xenopus shisa-2* (GenBank accession no. DQ342341) contains an open reading frame of 867 nucleotides encoding a 288 amino acid protein with a predicted molecular mass of 31.1 kDa. Similarly to *Xenopus* Shisa, Shisa-2 contains a signal peptide, two conserved cysteine-rich domains (CRD) in the amino-terminal half and a putative transmembrane domain N-terminally to the second CRD. The predicted amino acid sequence of *X*shisa-2 has close similarity to *X*shisa (Identity = 30.7%, Positives = 46.3%; Fig. 1A). Comparison of *Xenopus* Shisa and Shisa-2 sequences with that of mouse Shisa reveals higher conservation between *X*shisa-2 and mouse Shisa (I = 76.9%, P = 84.4%) than between *X*shisa and mouse Shisa (I = 31.9%, P = 45.2%; Fig. 1A), suggesting that *X*shisa-2 is the true ortholog of the previously described mouse protein.

The temporal expression of *Xshisa*-2 was analyzed by RT-PCR using total RNAs isolated from different developmental stages (Fig. 1B). Transcripts encoding *Xshisa*-2 are present in all stages analyzed (from mature oocyte until stage 48) and its expression is upregulated in neurula and early tailbud stages.

The spatial expression of *Xshisa-2* during early *Xenopus* development was characterized by whole-mount *in situ* hybridization. *Xshisa*-2 transcripts were not detectable by *in situ* hybridization until gastrulation. At late gastrula/early neurula stages *Xshisa-*

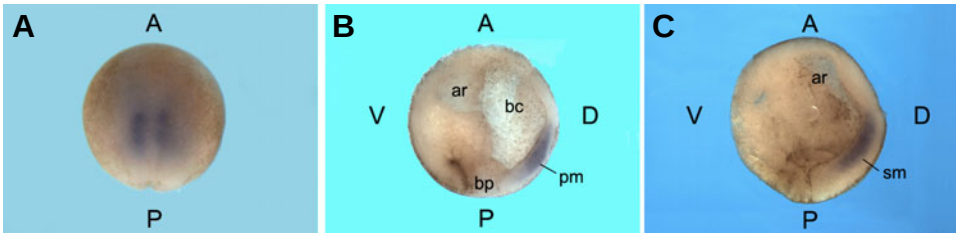

Fig. 2. *Xshisa-2* expression in the end of gastrulation and beginning of neurulation. *Whole mount* in situ *hybridization with a Shisa-2 DIG-labeled antisense RNA probe was performed on embryos at the end of gastrulation and beginning of neurulation.* **(A)** *Xshisa-2 expression by the end of gastrulation (st 13) is restricted to two narrow stripes on the dorsal side of the embryo, but not in the notochord. Posterior dorsal view.* **(B-C)** *Hemisections of stage 12 and 14 embryos show that Xshisa2 mRNA is restricted to the posterior portion of the paraxial mesoderm. Hemisections with dorsal to the right. A, anterior; P, posterior; D, dorsal; V, ventral; ar, archenteron; bc, blastocoel; pm, paraxial mesoderm; sm, somitic mesoderm.*

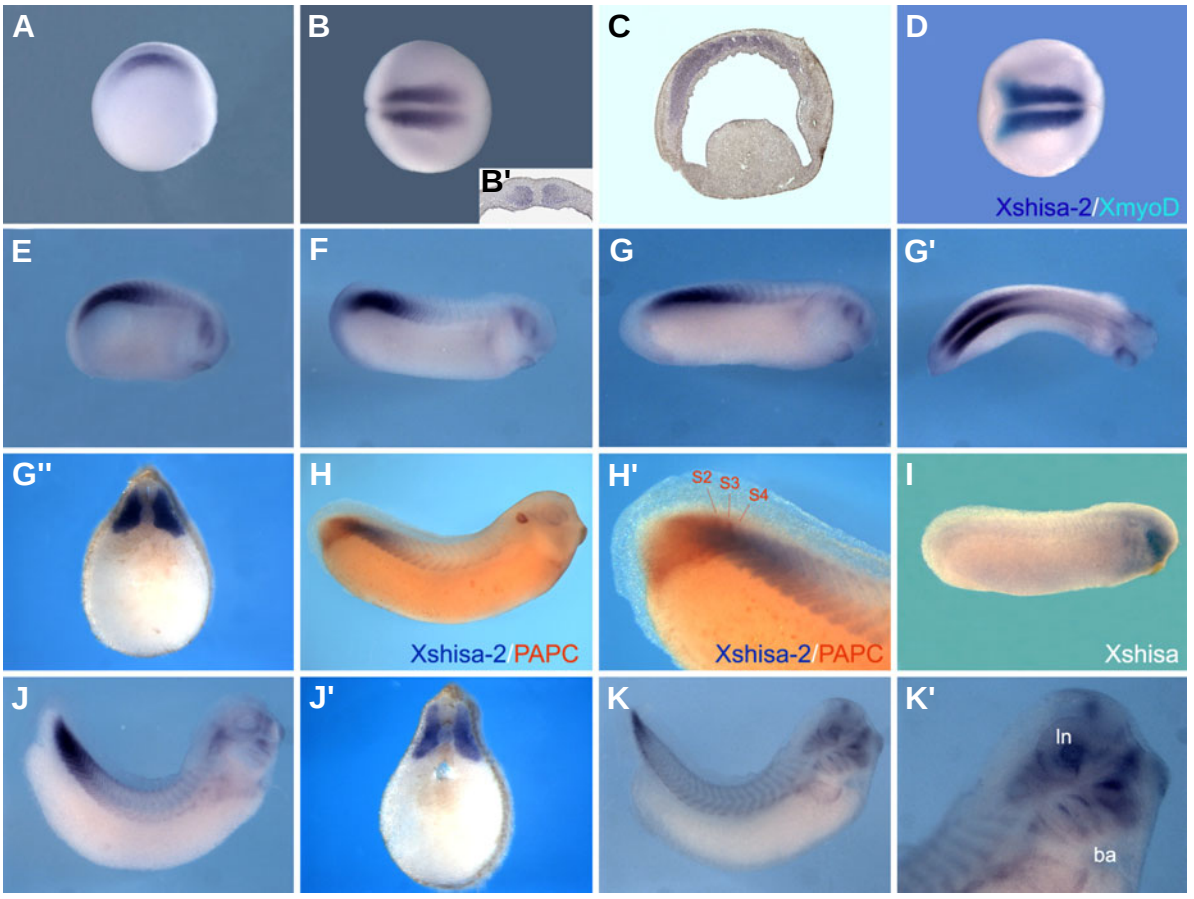

Fig. 3. Expression pattern of *Xshisa-2* during tailbud stages. From early tailbud stage onward, a dynamic pattern is observed in the forming somites. (A-B) *At stage 18, Xshisa-2 becomes progressively reduced in the anterior paraxial mesoderm.* **(B')** *Transverse section showing Xshisa-2 expression in the entire somite region.* **(C)** *Parasagittal section of a stage 18 embryo.* **(D)** *Double whole mount* in situ *hybridization with Xshisa-2 DIG-labeled antisense RNA probe and XmyoD fluo-labelled antisense RNA probe. The expression domain of XmyoD extends more posteriorly than the Xshisa-2 expression domain.* **(E-H,J-K)** *Expression of Xshisa-2 is stronger in the presomitic mesoderm and decreases as somites form. Transverse sections through the trunk region of stage 27* **(G'')** *and 30* **(J')** *embryos show Xshisa-2 expression in the entire somite.* **(H,H')** *Double whole mount* in situ *hybridization with Xshisa-2 and PAPC shows that Xshisa-2 is not expressed in the unsegmented region of the presomitic mesoderm.* **(I)** *Whole mount* in situ *hybridization with Xshisa shows expression restricted to the head region, a distinct expression pattern than the one observed for Xshisa-2.* **(K')** *A complex expression pattern is also observed in the head, including the lens (ln) and branchial arches (ba). (A,C,E-G,H-J,K,K') Lateral view. (B,D,G'). Dorsal view. All embryos are oriented with anterior to the right.*

2 is restricted to two stripes in the dorsal side but excluded from the dorsal midline (Fig. 2A). A sagittal section shows that the cells expressing *Xshisa*-2 are in the posterior portion of the paraxial mesoderm, but not in the neuroectoderm (Fig. 2 B,C). As somitogenesis commences, *Xshisa*-2 transcripts localize to a paraxial region, lateral to the involuting neural tube (Fig. 3B). As development proceeds, a dynamic expression pattern is observed in forming somites (Fig. 3). Its expression is stronger in the presomitic mesoderm and decreases as somites are formed. *XmyoD* is expressed in all myogenic cells throughout somitogenesis (Hopwood *et al.*, 1989). *Paraxial protocadherin* (*PAPC*) expression in the PSM is restricted to the anterior halfs of somitomeres 2, 3 and 4 and is expressed uniformly from somitomere 1 to the unsegmented portion of the PSM, the tailbud domain (TBD; Kim *et al.*, 2000). Comparing *Xshisa*-2, *XmyoD* and *PAPC* expression pattern one can observe that *XmyoD* and *PAPC* domains extend more posteriorly than the *Xshisa-2* expression domain (Fig. 3 D,H,H') which ends after the more posterior *PAPC* segmented stripe (S2) and is not expressed in the unsegmented portion of the presomitic mesoderm. A transverse section of the trunk region of a stage 27 embryo shows *Xshisa*-2 expression in the entire somite (Fig. 3G''). From early tailbud stages onwards *Xshisa*-2 is also expressed in the developing eye region. A complex expression pattern is also observed in the head at late tailbud stages, including the lens, the branchial arches (Fig. 3K'). Unlike *Xshisa-2*, *Xshisa* is expressed during tailbud stages exclusively in the head region (Fig. 3I).

In this work, we report the isolation and developmental expression pattern of a second *Xenopus shisa* gene, *Xshisa-2*. The founding member of this increasing family, *Xshisa*, was reported to exert its activity through a novel mechanism by which both the Wnt and the FGF signaling pathways are inhibited. This activity was used by *Xshisa* to pattern the anterior region of the *Xenopus* embryo. As reported here, *Xshisa-2*, a closely related member of

this family has an expression pattern opposite to that of *Xshisa*, at the level of the posterior mesoderm where it might be involved in formation/segmentation of the somites. The similarity to *Xenopus* Shisa raises the question of whether Xshisa-2 also functions as antagonists of Wnt and FGF signaling. Further biochemical and genetic analyses should help clarify the biological function of *Xshisa-2* during embryonic development. Taken together, this family of genes might be employing the same strategy, inhibition of the maturation of Wnt and FGF receptors, to pattern both the anterior and the posterior regions of the *Xenopus* embryo.

Experimental Procedures

Xenopus *embryo manipulations*

Xenopus eggs were obtained from females injected with 300 IU of human chorionic gonadotrophin (Sigma) and were fertilized *in vitro*. Eggs were dejellied with 2% cysteine hydrochloride pH 8. Embryos were grown in 0.1XMBS-H (1X MBS-H = 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.82 mM MgSO4, 0.41 mM CaCl2, 0.33 mM Ca(NO3)2, 10 mM HEPES pH 7.4, 10 μg/ml streptomycin sulfate and 10 μg/ml penicillin) and staged according to Nieuwkoop and Faber (1967).

Cloning of Xenopus shisa-2

The EST containing the *Xenopus laevis shisa-2* partial coding sequence, GenBank Acc. BJ042155, was obtained from NIBB (clone XL050n07; http://xenopus.nibb.ac.jp/).

To isolate the full length *Xshisa-2* coding sequence, total RNA from late neurula (stage 18) *Xenopus laevis* embryos (Nieuwkoop and Faber, 1967) was isolated using Trizol® reagent (Invitrogen) according to the manufacturer's protocol. First strand cDNA was synthesized with H minus M-MuLV reverse transcriptase (Fermentas) using random hexamers as primers. The following primers were used to amplify the *Xshisa-2* gene product by PCR:
Forward, 5'-TTTATCGATATGTGGTTGGAGGGCTCCCCCCTG -3';
Reverse, 5'- TTTCTCGAGCTACACAGTCACGGCTGGGTACATC -3', 65°C, 25 cycles). The PCR product was cloned into pCRII-TOPO® (Invitrogen). The sequence of *Xshisa-2* cDNA described here has been deposited in GenBank under accession number DQ342341.

Whole mount in situ *hybridization and histology*

Single and double whole mount *in situ* hybridization and anti-sense probe preparation was carried out as previously described (Belo et al., 1997; Epstein et al., 1997). Digoxigenin-labeled Xshisa-2 antisense RNA probe was synthesized by linearizing the XL050n07 clone (pBS(SK)Xshisa-2) using XbaI and transcribing using T7 RNA polymerase. The probe was then partially fragmented for 6 min at 60ºC in hydrolysis buffer [40mM $NaHCO_3$, 60mM Na_2CO_3, pH 10.2] followed by sodium acetate/ethanol precipitation. To generate the fluorescein labeled XmyoD and PAPC antisense RNA probes, plasmids containing XmyoD and PAPC fragments were linearized using HindIII and XbaI respectively and transcribed using T7 RNA polymerase. Stained embryos (stage 18 and above) were bleached by illumination in 1% H_2O_2, 4% formamide and 0.5xSSC pH 7.0.

For histology, *Xenopus* embryos previously *in situ* hybridized were fixed ON at 4˚C in 4% PFA, embedded in gelatin and sectioned to 16mm with a cryostat.

RT-PCR

Total RNA was prepared from pools of 5 embryos with Trizol® reagent (Invitrogen) according to the manufacturer's protocol. First strand cDNA primed by random hexamers was synthesized with H minus M-MuLV reverse transcriptase (Fermentas) and PCR was performed using standard conditions and the following sets of primers:
shisa-2-F (5'-TCCTTCTCTCAGTGCTGGCG-3') and
shisa-2-R (5'-ATCGGGACTGTCCTTGTCCG-3'), 55°C, 25 cycles;
ODC-F (5'-CAGCTAGCTGTGGTGTGG-3') and
ODC-R (5'-CAACATGGAAACTCACACC-3'), 57°C, 21 cycles.

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