Int. J. Dev. Biol. 52: 81-85 (2008)
doi: 10.1387/ijdb.072355th

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Expression of *Shisa2*, a modulator of both Wnt and Fgf signaling, in the chick embryo

THOMAS A. HEDGE and IVOR MASON*

MRC Centre for Developmental Neurobiology, King's College London, Guy's Campus, London, U.K.

ABSTRACT Shisa proteins are a recently-identified family of modulators of both FGF and Wnt signaling that block both maturation and transport to the cell surface of their respective receptors. The latter are retained within the endoplasmic reticulum, thereby inhibiting or reducing cellular responses to the ligands. We describe expression of a *Shisa2* orthologue in an amniote: the chick embryo. We show that *Shisa2* transcripts are expressed in a dynamic manner along the antero-posterior axis, in a manner consistent with a role in head development as demonstrated for *Xenopus* Shisa, being ubiquitously expressed in anterior tissues. However, expression is progressively restricted anteriorly within the developing neural tube and adjacent mesenchyme and ectoderm, eventually becoming restricted to the telencephalic lobes. Similarly, from being ubiquitous within the optic cups, transcripts become restricted to the prospective ciliary margin. A similar process is evident in the somites, where expression is initially ubiquitous but remains at high levels first in dermamyotome and subsequently is only detected in myotome. During the initial stages of organogenesis, *Shisa2* transcripts are detected in cardiac and lung bud mesenchyme and in nephric ducts and tubules. Within the pharyngeal region, expression is observed in pharyngeal pouches from their first appearance and later in mesenchyme of all pharyngeal arches, as well as in cranial ganglia. Transcripts are also detected in the dorsal mesenchyme of the limb bud.

KEY WORDS: *endoplasmic reticulum, posteriorisation, ciliary marginal zone*

Members of the Wnt and FGF families of secreted signaling proteins regulate multiple developmental processes, frequently being deployed together to instruct morphogenesis (Logan and Nusse, 2004, Thisse and Thisse, 2005). A feature of Wnt, FGF and other signaling pathways is the utilisation of extracellular and intracellular modulators, which serve to control the duration, strength and spatial extent of their action. Most commonly these are antagonists, often part of negative feedback loops, although some serve to amplify the signals (Dailey *et al.*, 2005, Kawano and Kypta, 2003, Logan and Nusse, 2004, Tsang and Dawid, 2004). However, a novel regulatory mechanism, mediated by an endoplasmic reticulum-resident protein, Shisa, has recently been identified in *Xenopus* (Yamamoto *et al.*, 2005). Shisa regulates the post-translational modification and trafficking to the cell surface of receptors for both Wnts (frizzled proteins; Fz) and FGFs (FGFRs). Inhibition studies in that organism revealed an early essential role for Shisa in head development through its suppression of responses to these caudalising factors (Yamamoto *et al.*, 2005). A recent study has shown that all vertebrates have at least four *Shisa* (*Shisa2-5*) genes in their genomes, with zebrafish and *Xenopus* having an additional member; the prototypic *Shisa* (now *Shisa1*) (Furushima *et al.*, 2007). The biochemical studies described above were performed using the latter, however murine Shisa2 has been shown to regulate Wnt and FGF activity in a similar manner to *Xenopus* Shisa1 (Furushima *et al.*, 2007).

Our interrogation of the first draft of the chicken genome identified a single *Shisa* orthologue on chromosome 6 within contig 9.365. Sequences from that region were used to identify a chicken cDNA in the Genbank database (accession number NM_204501), a corresponding EST was obtained and identified as the chicken *Shisa2* orthologue (Furushima *et al.*, 2007). This EST was used to characterize *Shisa2* expression between gastrulation and limb bud stages of development (Fig. 1 A-R, Fig. 2 A-P).

At the earliest stage examined, stage 4 of Hamburger and Hamilton (1951; HH4), *Shisa2* mRNA was detected in the anterior

***Address correspondence to:** Ivor Mason. MRC Centre for Developmental Neurobiology, 4th Floor New Hunt's House, King's College London, Guy's Campus, London, SE1 1UL, U.K. Tel: +44-(0)-207-848-8148. Fax: +44-(0)-207-848-6798. e-mail: ivor.mason@kcl.ac.uk
Website: http://www.kcl.ac.uk/depsta/biomedical/mrc/ResearchGroup.php?GroupID=20

Accepted: 18th July 2007. *Published online:* 13th November 2007.

0214-6282/2008/$35.00

Printed in Spain

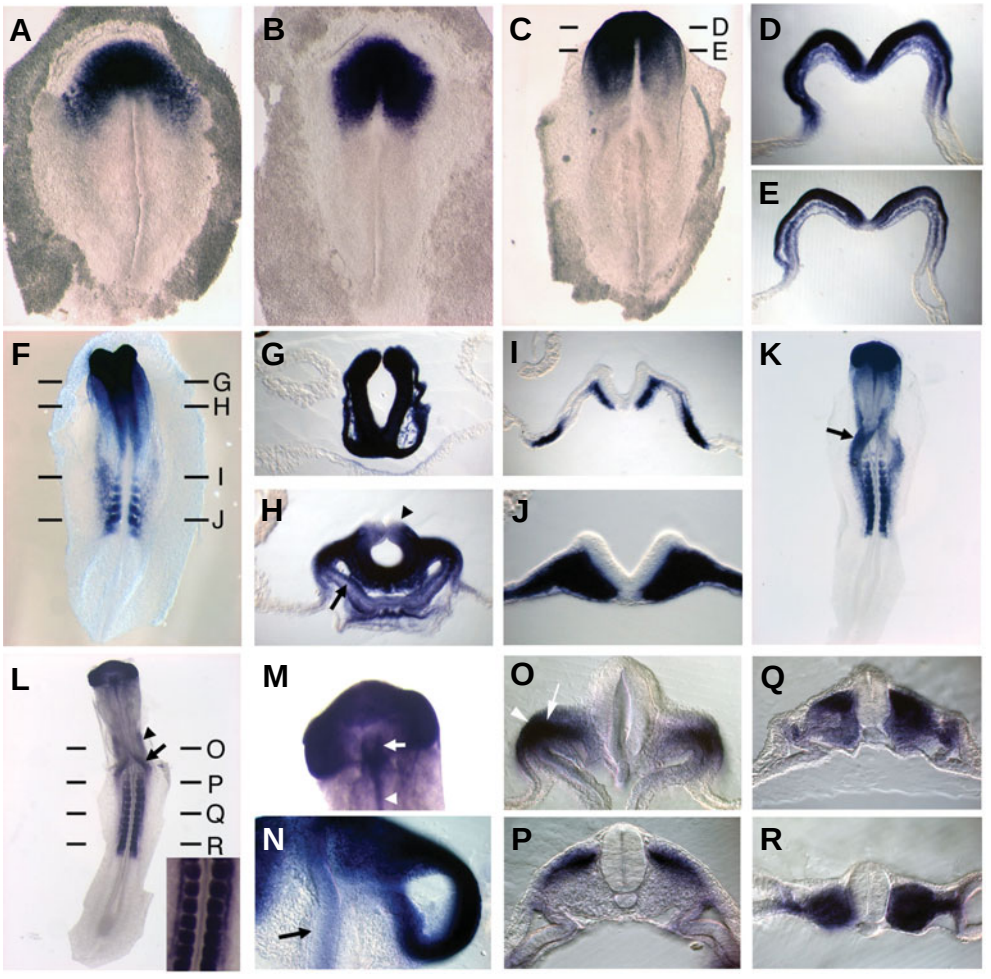

Fig. 1. *Shisa2* expression between gastrulation and neural segmentation stages. In situ *hybridisation to embryos at* **(A)** *HH4,* **(B)** *HH5,* **(C-E)** *HH6,* **(F-J)** *HH8,* **(K)** *HH10 and* **(L-R)** *HH12. (A-C,F,K-M) Whole embryo preparations.* **(D,E)** *Transverse sections of an HH6 embryo taken at the levels indicated in* **(C)**. **(G-J)** *Transverse sections of an HH8 embryo taken at the levels indicated in* **(F)**. *Reduced abundance of* Shisa2 *transcripts in dorsal neural folds and ventral endoderm and ectoderm are indicated in* **(H)** *by an arrowhead and arrow respectively. Arrow in* **(K)** *indicates cardiac mesenchyme in an embryo viewed ventrally.* **(L)** *Inset: higher magnification of trunk region to show expression in intermediate mesoderm adjacent to somites. Arrowhead indicates transcripts in the pharyngeal region, arrow indicates expression in cardiac mesenchyme.* **(M)** *Higher magnification image of the anterior region of the embryo in (L); arrow indicates prechordal mesoderm and arrowhead indicates the head process.* **(N)** *Longitudinal section through an optic lobe; reduced mRNA levels in the posterior diencephalon are indicated (arrow; see also Fig. 2B) while transcripts are undetectable in mesenchyme posterior to the optic lobe.* **(O-R)** *Transverse sections of an HH12 embryo taken at the levels indicated in (L) Arrowhead indicates ectoderm and arrow indicates mesoderm associated with pharyngeal pouches in (O).*

of the embryo (Fig. 1A) and this pattern of expression persisted during early stages of node regression (Fig. 1 B,C). Sections of embryos at these stages showed the presence of transcripts in all three germ layers, with expression being particularly prominent in the ectoderm and neural plate (Fig. 1 D,E). Comparison with fate maps of the neural plate at HH4 (Cobos *et al.*, 2001) indicated that transcripts were present in prospective forebrain, midbrain and hindbrain and, overall ectodermal expression was most reminiscent of *Otx2* at that stage (Bally-Cuif *et al.*, 1995, Puelles *et al.*, 2005) and complementary to that of *Gbx2* (Shamim and Mason, 1998). Thereafter, expression in the neural plate and neural tube was dynamic and characterized by a progressive rostral shift in the caudal limit of *Shisa2* expression. A recent study of mouse *Shisa2* expression also reported that it was co-extensive with *Otx2* at neural plate stages. Moreover ectopic expression in *Xenopus* resulted in expansion of the *Otx2* domain and *Shisa2* transcripts were reduced or absent in *Otx2* null mice (Furushima *et al.*, 2007).

During early neurulation stages mRNA was detected in a domain that is fated to give rise to forebrain and midbrain, consistent with co-expression with *Otx2* (Fig. 1F; (Couly and Le Douarin, 1985); in anterior regions transcripts were present throughout the neuroepithlium and in all other tissues at uniformly high levels (Fig. 1G). However, more posteriorly, reduced levels of expression were observed in the dorsal neural tube and in ventral ectoderm and endoderm lining the floor of the pharynx (Fig. 1H).

As the neuromeric architecture of the developing brain became apparent at HH10 (10 somites), *Shisa* transcripts were further restricted to the prospective diencephalon and telencephalon and their adjacent ectoderm. Transcripts were also detected in anterior head process and prechordal mesoderm and, at lower levels, in mesenchyme adjacent to the midbrain (Fig. 1K and data not shown). Approximately 12 hr later (HH12), expression remained elevated in the optic lobes and prospective telencephalon while becoming reduced in the posterior diencephalon, although abundant transcripts were still detected in underlying prechordal mesoderm and anterior head process (Fig. 1 L-N). Transcripts were now undetectable in head mesenchyme posterior to the optic cups (Fig. 1N). Shortly thereafter *Shisa2* RNA became restricted within the brain to the telencephalon and optic lobes (Fig. 2 A,B). As development proceeded, there was further apparent reduction in transcript levels within the telencephalon (Fig. 2 D,E,L), such that by HH21 highest RNA levels were detected in the lateral telencephalic vesicles (Fig. 2 L,M).

Within the developing ocular system, abundant transcripts were detected in the developing retina and lens but became reduced in the optic stalks (HH15; Fig. 2 D,F). However, at later stages (HH19, 21) apparent expression was confined to the prospective ciliary marginal zone, a source of retinal stem cells in

the chick (Kubota *et al.*, 2004, Moshiri *et al.*, 2004) and was undetectable throughout the retina or within the lens (Fig. 2 E,G,L,O). Notably, both FGF and Wnt signaling has been implicated in regulation of retinal development and regeneration at the ciliary margin (Liu *et al.*, 2007, Spence *et al.*, 2004). In the oldest embryos examined, apparent, weak expression was detected in Rathke's pouch (data not shown) and in cranial ganglia (Fig. 2L; data not shown) but not in other components of the peripheral nervous system.

Somites are subject to instructive signaling by both FGFs and Wnts (see e.g. Brent and Tabin, 2004, Linker *et al.*, 2005 and references therein) and *Shisa2* transcripts were detected in developing somites from the onset of somitogenesis (Fig. 1 F,K, L, Fig. 2 A,D,E,L) and, as in the brain, exhibited a dynamic pattern of expression during their maturation. Initially, RNA was detected throughout recently-cleaved, epithelial somites (Fig. 1 J,R). As maturation proceeded, transcripts became progressively restricted, first to the dermamyotome (Fig. 1 P,Q, Fig. 2 C,P) and eventually to the myotome only (Fig. 2I and data not shown). At early stages, expression was also found in lateral plate mesoderm (Fig. 1 I,J) but this did not persist (see e.g. Fig. 1P). Within the head at HH19, transcripts were detected medial to the trigeminal ganglion in cells identified by others as the precursors of the lateral rectus muscle of the eye at that stage of development (Fig. 2 E,H) (Mootoosamy and Dietrich, 2002).

Within the posterior of the embryo *Shisa2* mRNA was undetectable within the primitive streak, tail bud and segmental plate at all stages (Fig. 1 A-C,F,K,L; Fig. 2 A,D,E,L) and, while expression was detected anteriorly within the head process and prechordal mesoderm (see above), transcripts were never detected in the notochord (Fig. 1 J, P-R, Fig. 2 H,I,K,P). *Shisa2* was observed in cardiac mesenchyme at early somitogenesis stages (Fig. 1 K,L) and later in mesenchyme in the region of the developing lung buds (Fig. 2 A,D,E,J,L). Transcripts were also detected in intermediate mesoderm (Fig. 1 L,Q) and subsequently in nephric ducts (Fig. 2C) and mesonephric tubules (Fig. 2N). At early stages of limb bud outgrowth, expression was undetectable in the buds (Fig. 2K), however at the oldest stages analysed, RNA was detected in the dorsal mesenchyme (Fig. 2P) a region known to be subject to Wnt signaling (Kengaku *et al.*, 1998).

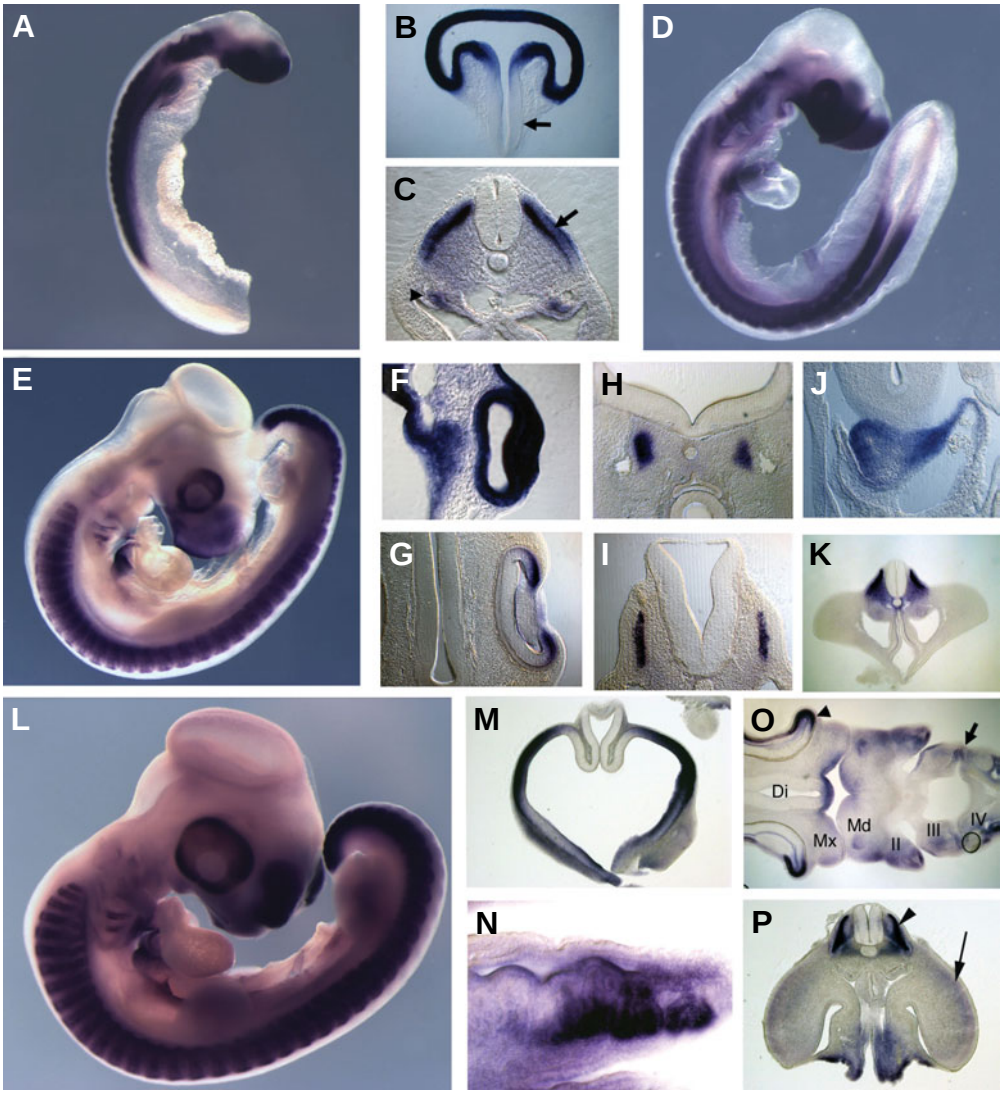

Fig. 2. *Shisa2* expression between flexure and limb bud stages. In situ *hybridisation to embryos at* **(A-C)** *HH13,* **(D,F)** *HH15,* **(E, G-K)** *HH19 and* **(L-P)** *HH21.* **(A,D,E,L)** *Whole embryo preparations.* **(B)** *Coronal section through the forebrain, anterior to the top; note lack of detectable transcripts in the diencephalic tissue (arrow).* **(C)** *Transverse section through an anterior somite showing elevated expression in dermamyotome (arrow) compared to adjacent sclerotome. Arrowhead indicates transcripts associated with the nephric duct.* **(F,G)** *Coronal section taken through the retina and lens at HH15 (F) and HH19 (G).* **(H)** *Transverse section showing expression in cells at the position of precursors to the lateral rectus muscle of the eye.* **(I)** *Transverse section taken at the level of the posterior hindbrain showing expression restricted to the myotome.* **(J)** *Mesenchymal expression in the region of the developing lung buds.* **(K)** *Transverse section at the level of the wing bud.* **(M)** *Coronal section taken through the telencephalon.* **(N)** *Longitudinal section showing expression in tubules of the mesonephros.* **(O)** *Longitudinal section taken through the pharyngeal region. Arrowhead indicates expression in the prospective ciliary body and arrow indicates expression in the third pharyngeal pouch. Abbreviations: Di, diencephalon; Md, mandibular process; Mx, Maxilliary process; II,III,IV, second, third and fourth pharyngeal arches respectively.* **(P)** *Transverse section taken at the level of the hindlimb bud. Arrowhead indicates dermamyotome and arrow indicates dorsal limb bud mesenchyme.*

Transcripts were detected within the pharyngeal region adjacent to the developing hindbrain (HH12; Fig. 1L), a prominent site of Fgf expression in the avian embryo (see e.g. Mahmood *et al.,* 1995; Shamim and Mason 1999; Shamim *et al.,* 1999a) and were particularly abundant in the mesenchyme and ectoderm adjacent to the pharyngeal pouches (Fig. 1O). Expression persisted in the pouches at later stages (Fig. 2 E,L,O) and was also detected in mesenchyme of the maxilliary and mandibular lobes of the first pharyngeal arch and in all posterior arches (Fig. 2O).

In summary, *Shisa2* transcripts were detected in many tissues that are known to be influenced by FGF and Wnt signaling. Our findings are similar but not identical to those reported by others for avian *Shisa2*, although that study was not as detailed as this one, particularly at later stages (Filipe *et al.*, 2006). A brief report of expression of the mouse *Shisa2* orthologue at similar stages to those used in our study indicates that its pattern of expression is conserved among amniotes (Furushima *et al.*, 2007). By contrast, expression of the *Xenopus Shisa1* orthologue seems more similar to amniote *Shisa2* than the *Xenopus Shisa2* cognate (Silva *et al.*, 2006).

It is noteworthy that our study reveals that the relationship between *Shisa2* expression and Wnt- or FGF-dependent processes is not absolute, for example it does not appear to be associated with gastrulation movements, initial stages of limb bud outgrowth, otic induction or patterning signals from the mid-hindbrain boundary; processes regulated by both types of ligand. Furthermore, it cannot be considered to be part of a synexpression group for either family, unlike many other antagonists. The studies of murine *Shisa3-5* published to date indicate that this is not due to differential sub-functionalisation during evolution (Furushima *et al.*, 2007).

Axial expression from earliest stages is consistent with the proposed role for the Shisas in antagonizing posteriorizing reponses to FGF and Wnt signals in *Xenopus* (Yamamoto *et al.*, 2005) and progressive restriction to anterior regions indicates that competence to respond to them is temporally modulated. Likewise, dynamic spatial and temporal expression during somitic maturation and differentiation is indicative of altering responses to extracellular cues during their development. However, mice homozygous for a targeted null mutation in *Shisa2* have normal early development, although the majority are reduced in size at birth. The authors argue against compensation by other *Shisa* genes, although other mechanisms are possible (Furushima *et al.*, 2007).

Experimental Procedures

A chicken *Shisa2* orthologue was identified by interrogation of the first draft release of the chicken genome (http://www.ensembl.org/Gallus_gallus/index.html) using the TBLASTN algorithm. The sequences recognized were used to identify a chicken EST (ChEST994o2) in the BBSRC ChickEST database (http://www.chick.umist.ac.uk/), which was then obtained through Geneservice (http://www.geneservice.co.uk/). Embryos were staged according to (Hamburger and Hamilton, 1951) and *in situ* hybridization was performed as described (Henrique *et al.*, 1995). Embryos were embedded in gel albumen and sectioned on a vibrotome as described (Shamim *et al.*, 1999).

Acknowledgements

This work was supported by grants from the Medical Research Council and The Wellcome Trust. TAH is supported by an MRC studentship award and IM is a Research Fellow of the Leverhulme Trust.

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