Investigation of mRNA Expression for Secreted Frizzled-Related Protein 2 (sFRP2) in Chick Embryos

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Abstract. The roles of secreted frizzled-related protein 2 (sFRP2) in organ development of vertebrate animals are not well understood. We investigated expression of sFRP2 during embryogenesis of Arbor Acre broiler chicken eggs. Expression of sFRP2 was detected in the folds and lateral layer of developing brains. The sFRP2 signals in the developing eye were marked as a circle along the orbit. In younger embryos on days 3–6, the sFRP2 signals were consistent with growth of the sclerotome, suggesting that sFRP2 may be associated with somite development. Furthermore, with the exception of bones, sFRP2 mRNA was detectable in the interdigital tissue of embryos older than eight days as the limbs matured. This revealed that sFRP2 might play a role in myogenesis. In situ hybridization was also used to analyze the expression of sFRP2 in day 3–10 chick embryos. Signals were expressed in the gray matter of the developing brain coelom, including the optic lobe, metencephalon, myelencephalon, mesencephalon and diencephalon. The developing eyes contained an intercellular distribution of sFRP2 in the pigmented layer of the retina and photoreceptors. Furthermore, sFRP2 was expressed in the mantle layer of the neural tube and notochord. Based on these findings, it seems reasonable to suggest that sFRP2 may play an active role in embryogenesis, especially in development of the neural system, eyes, muscles and limbs.

Key words: Chick, Development, Embryo, Secreted frizzled-related protein 2 (sFRP2)  
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between stages 8 and 24. During chick embryogenesis, sFRP1 is distributed in the anterior primitive streak, isthmus, telencephalic vesicle, epiphysis, ventral hindbrain, neural tube, otic placode, notochord, dorsal root ganglion, sympathetic ganglion, mesoderm of the branchial arch, developing eye, interphalangeal joint, pharynx, esophagus, gut, lung bud, liver, heart, facial bone and urogenital system [16–18]. It can also be detected in developing neural structures, differentiated neurons, facial primordia, limb buds, trunk, the heart, and developing eyes [16, 19]. The major sites of sFRP2 expression in murine embryos are developing neural structures and the regions of myogenesis. The fact that sFRP2 physically interacts with Wnt-4 suggests that it modulates (inhibits) Wnt-4 signaling in the metanephric mesenchyme [9]. The sFRP2 protein is eliminated from developing eyes in murine embryos at a later stage of development [20]. Expression of sFRP3 mRNA is found in the neural crest of the oculomotor nerve, sclerotome cells of the myotome and notochord, adrenal cortex, gonad, circulating blood cells, cartilage of the spine and limbs, neural epithelium of the prosencephalon, mesencephalon and rhombencephalon, neural tube, sympathetic ganglion, dorsal root ganglion, and heart in chicks and mice [20, 21]. Expression of sFRP4 is found in the kidney, dense outer cell and inner cell layers of the neural retina, incisor teeth, and salivary gland of the adult mouse. It is also expressed in human cardiomyocytes and the stroma of the proliferative endometrium of the *Xenopus* [14, 20, 22]. Expression of sFRP5 has been detected in the retinal pigment epithelium of the *Xenopus* and pancreas, heart, muscle, liver and retina pigment epithelium in humans [23]. The aim of this study was to investigate sFRP2 mRNA expression in developing chick embryos by *in situ* hybridization.

**Materials and Methods**

**Chick embryos**

Arbor Acre broiler chicken eggs were incubated at 38 C in a humidified incubator. Embryos were staged according to previously published reports [24].

**Isolation and amplification of csFRP2 cDNAs**

The HH stage 11 to 36 embryos were harvested and homogenized for extraction of total RNA using TriZOL® reagent (Gibco, Carlsbad, CA, USA). Two µl total RNA was reverse-transcribed using 80 µg/ml Oligo (dT)12–18 (Gibco) and 20 U M-MLV transcriptase (Gibco). The reaction was incubated at 42 C for 50 min. The reverse transcriptase was inactivated, and the template-cDNA complexes were denatured by heating the reaction to 70 C for 15 min. The chick sFRP2 cDNA was amplified by PCR using oligonucleotide primers for the 5' ORF sequence, CCG CCA TGA CGC GCC GC CTC, and 3' ORF sequence, CCA AAG CTT CTA ACA CTG CAG T [8]. The internal control gene (glyceraldehyde 3-phosphate dehydrogenase: GAPDH), was amplified using the following primers: 5'-ACG CCA TCA CTA TCT TCC AG-3' (upstream) and 5'-CAG CCT TCA CTA CCC TCT TG-3' (downstream). GAPDH; GenBank accession number: K01458. The PCR program consisted of 35 cycles of three 1-min steps at 95, 60 and 72 C. The full-length sFRP2 fragment was then subcloned into the plasmid pCRII (Invitrogen), and its orientation was determined by nucleotide sequencing.

**In vitro transcription**

The EcoRI fragment of the constructed plasmid encoded nucleotides 20 to 912 of sFRP2. This region encodes the full length of the sFRP2 protein. The digoxigenin (DIG)-labeled sFRP2 antisense cRNA probe for dot blotting analysis and *in situ* hybridization was prepared with SP6 RNA polymerase (Roche, Mannheim, Germany) using an EcoRV-digested plasmid. The pCRII/sFRP2 was linearized by SacI and transcribed with T7 RNA polymerase (Roche), to generate a sFRP2 sense cRNA probe as a control for non-specific hybridization of *in situ* hybridization.

**Northern blotting hybridization**

Fifteen micrograms of total RNA was denatured with 5 × MOPS buffer, 37% formaldehyde, and 50% deionized formamide at 65 C for 15 min and was then chilled on ice for 5 min. The RNA sample was mixed with 5 × formaldehyde gel loading buffer. Electrophoresis was performed on 1.2% formaldehyde-agarose gel at 50 V for about 3 hr. The RNA was then transferred onto a nitrocellulose (NC) membrane (Whatman, Brentford, UK) and hybridized overnight with a DIG-labeled antisense sFRP2 probe at 42 C. The membrane was washed
and the positive signal was detected with a color-substrate solution (Roche).

In situ hybridization (ISH) of tissue sections
Embryos were dissected free from the extra embryonic membrane and fixed in 10% formamide in PBS$_{DEPC}$ for 24 h. The tissue section was dewaxed at 60°C for 30 minutes and then processed through an ethanol series. Treatment in 0.2 M HCl for 20 min and addition of 0.2 mg/ml glycine$_{PBS-DEPC}$ were used to stop the reaction. The tissue was digested in proteinase K (Promega, Madison, WI, USA) and then hybridized overnight with a 150 ng/ml DIG-labeled probe at 47°C. All in situ hybridizations were performed by adding anti-sense cRNA probe or sense cRNA probe (negative control) to respective sections from the same tissue sets for signal comparison. Positive signals were detected using NBT/BCIP solution (Roche).

Whole mount ISH
Embryos were washed, fixed in freshly made 4% paraformaldehyde in PBS$_{DEPC}$, and rehydrated through a methanol series. The embryo was digested in 10 μg/ml proteinase K for 45–60 min. It was then post-fixed for 20 min in 4% paraformaldehyde and washed with PTW. Whole mount in situ hybridization was carried out in a 2 ml eppendorf tube with 200 ng/ml DIG-labeled sFRP2 probe at 70°C overnight. Anti-sense sFRP2 cRNA probe and sense probe (negative control) were added into paired eppendorf tubes with embryos, respectively, for signal comparison. The embryo was then washed several times in hybridization buffer and TTBS. Blocking solution was added for 3 hr with gentle shaking. Positive signals were detected with NBT/BCIP solution in a dark room.

Results

Northern blotting
In order to understand the dynamic expression of sFRP2 mRNA during chick embryo development,
day 3–10 embryos were collected and RNA was extracted. The specificity and sensitivity of the DIG-labeled antisense and sense probes were verified by dot hybridization (data not shown). The abundance of sFRP2 mRNA elevated with embryo growth during days 3–10 (Fig. 1A).

ISH
ISH using the DIG-labeled sense probes (negative controls) corresponding to the antisense probes failed to produce a signal for expression of sFRP2 mRNA in the developing chicken embryos (Fig. 2). The distributions of sFRP2 mRNA in developing tissues and whole mount sections from days 3–10 obtained using ISH is systematically described below.

**Expression of sFRP2 in the developing brain**
Strong positive signals were detected for sFRP2 around the brain vesicle in whole mount sections of embryos on days 3–10. On day 6, signals were evident in the rhombencephalon and myelencephalon ventricles (Fig. 3A). Observation became difficult beginning on day 8 because of decreased transparency. Expression of sFRP2 mRNA was present in the ventral margin of the telencephalon ventricle, dorsal and lateral margins of the optic lobe, and diencephalons (Fig. 3B). On
Expression of sFRP2 in the developing eye. Whole mount ISH revealed clear positive signals in the optic vesicle (A and B). Expression of sFRP-2 was detected in the pars optica retinae and tissue around the eyes beginning on day 4 (D–H). Expression was observed in the ganglion cells of the neural retina beginning on day 7 (I–L). NR: Neural retina. PR: Pigmented retina. The arrows indicate locations with positive signals by in situ hybridization. Scale bars=200 µm.

day 10, expression of sFRP2 in the telencephalon and optic lobe shifted anteriorly (Fig. 3C). In tissue sections, positive signals were detected for sFRP2 in the optic lobe from day 3 (Fig. 3E) to day 10. Expression of sFRP2 mRNA was present in the mesencephalon (Fig. 3F, day 4), rhombencephalon (Fig. 3G), glial cells (Fig. 3H), and neurons (Fig. 3M). There was a considerable amount of sFRP2 expression in the corpus striatum beginning on day 6 (Fig. 3I) and last until day 7 (Fig. 3J). Expression of sFRP2 in neurons lasted until day 10.

Expression of sFRP2 in the developing eye
Marked expression of sFRP2 was present in the eyes, hearts and neural tubes of day 3 embryos (Fig. 4A). The lens started to develop after formation of the inter-retinal space of the optic cup (data not shown). Whole mount ISH revealed positive signals of sFRP2 in the orbital tissues, brain vesicles, the heart, and spinal cord (Fig. 4B). There were no obvious signals in the negative control sections compared with the same sites in the positive tissue sections, which were darkly pigmented (Fig. 4C). There were positive signals in the inner layer of the neural retinas and inter-retinal spaces of day 4 embryos (Fig. 4D). Beginning on day 5, the inner and outer layers of the retina began to merge, and sFRP2 mRNA was
Fig. 5. Expression of sFRP2 mRNA during spinal cord development. A positive signal was detected along the entire spinal cord in day 4 embryos (A). ISH of tissue sections showed that sFRP2 was distributed in the ependyma of the neural tube, sclerotome, myotome (C), mantle layer and the ependyma of the spinal cord (D–H). On day 9, sFRP2 expression was decreased and was only detected in a small part of the ependyma of the spinal cord (I). NC: Notochord. NT: Neural tube. SC: Sclerotome. The arrows indicate locations with positive signals by *in situ* hybridization. Scale bars=200 µm.

Fig. 6. Expression of sFRP2 mRNA in the developing somite. Expression of sFRP2 was distributed in the sclerotome, myotome and neural tube (A). Expression was present occurred in the neural bundle, spinal nerve through the vertebral body (B–H), and notochord throughout the entire vertebrate column (I). DR: Dorsal root ganglion. Me: Metanepharon. My: Myotome. NC: Notochord. SC: Sclerotome. V: Vertebrate. The arrowheads indicate the area of the myotome and sclerotome, respectively. The arrows indicate locations with positive signals by *in situ* hybridization. Scale bars=200 µm.
expressed around the pars optica retinae and orbital tissues (Fig. 4E). Expression of sFRP2 in the ganglion cells of the inner layer of the neural retina lasted until day 10 (Fig. 4F, day 6; Fig. 4H and I, day 7; Fig. 4L, day 10).

Expression of sFRP2 in the developing spinal cord

Expression of sFRP2 mRNA was detected in the neural tube, which differentiated into the spinal cord beginning on day 3 (Fig. 5A, day 4; control Fig. 5B). The sFRP2 mRNA was distributed in the ependyma of the neural tube, notochord, sclerotome, and myotome (Fig. 5C) on day 3 and was also present in the mantle layer from day 4 (Fig. 5D and E) to day 8 (Fig. 4F and G). On day 9, sFRP2 expression was markedly reduced and could only be detected in a small part of the ependyma of the cranial spinal cord (Fig. 5I).

Expression of sFRP2E in the developing somite

Expression of sFRP2 was present in the sclerotome and myotome (Fig. 6A). After the sclerotome developed into the vertebral column, positive signals were no longer detectable for sFRP2. Beginning on day 5, expression of sFRP2 mRNA in the somite was distributed in the neural bundle near the dorsal vertebral column (Fig. 6C), spinal nerve (Fig. 6B), and neuron and myotome development areas (Fig. 6D and E). On day 9, there was some variation in development of the somite between the cranial (Fig. 6F) and dorsal sections of the vertebral column (Fig. 6G). However, differentiation of development did not appear to influence expression of sFRP2 mRNAs which was still observed in the spinal cord, spinal nerve, and muscle until day 10 (Fig. 6H and I).

Expression of sFRP2 in other developing tissues

Expression of sFRP2 mRNA was present ventrally around the facial structure of whole mount embryos from day 6 to 8 (Fig. 7A and B). Beginning on day 8, positive signals were detected
in the muscular tissues of the anterior limbs (Fig. 7C). The sFRP2 mRNA congregated in tissues proximal to the forelimb bones (Fig. 7E). Expression of sFRP2 was similar in the hindlimbs and forelimbs and was detectable in the joints (Fig. 7F). Positive signals were also present in the notochord (Fig. 7H) on days 3–10 and in the pharynx of day 3 embryos (Fig. 7I).

Discussion

Screening of the sFRP2 expression of developing chick embryos showed that it was dynamic during development of different organs. The expression pattern suggests that sFRP2 may be important in modulating these development processes. In two previous reports concerning chick embryos, expression of sFRP2 was mainly present during development of neuronal structures, muscles, and eyes [19, 25]. In the present study, we attempted to observe and understand sFRP2 expression during development of multiple organ systems in chick embryos on a day-by-day basis. Interestingly, the expression pattern and level of sFRP2 in the chick embryos varied between the different developing organ systems and different stages as described below.

Expression of sFRP2 in the developing brain

In the present study, sFRP2 was detectable in the ventricles of several brain tissues, including the rhombencephalon, myelencephalon, ventral margin of the telencephalon, dorsal and lateral margins of the optic lobe, and diencephalon. During later stages of development (days 8–10), sFRP2 expression in the brain regressed, which was consistent with the results of a previous study in the mouse [20]. The changes in expression suggest that sFRP2 is involved in earlier developmental processes in the brain. The expression of sFRP2 in the telencephalon ventricle and optic lobe shifted anteriorly in the present study, suggesting that the involvement of sFRP2 in development of the telencephalon ventricle and optic lobe switched from the ventral or dorsal side during the early stages to only the anterior area during the late stages. In another report concerning chick embryos, the expression of sFRP2 became regionalized along the anterior-posterior and dorsoventral axes of the neural tubes during later stages of expression [19].

Expression of sFRP2 in the developing eye

There was marked expression of sFRP2 in the developing eye of whole mount ISH and tissue sections, suggesting that sFRP2 has an important role in eye development, especially the development of the neural retina. Positive signals were observed in the inner neural retinas of day 4 embryos. Expression of sFRP2 in the inner neural retina suggests that sFRP2 has a role in its development instead of the outer retina during early ocular development. Beginning on day 5, sFRP2 mRNA was expressed around the pars optica retinae and orbital tissues. In one report concerning chick embryos, sFRP2 expression was reported in the neural retina and RPE, but no details were given regarding specific locations in the neural retina [25]. In other reports concerning canine retina, sFRP2 was abundantly expressed in the retina, especially the inner nuclear layer of the neural retina [26, 27]. Upregulated expression of sFRP2 mRNA has been found in the retina of humans with inherited retinal degeneration such as retinitis pigmentosa [28].

Expression of sFRP2 in the developing somite

Expression of sFRP2 was present in the chick sclerotome and myotome. It is interesting that positive signals were no longer detected for sFRP2 after the sclerotome developed into the vertebral column. This suggests that sFRP2 has an important role in developing the sclerotome. During later stages (beginning on day 5), the sFRP2 mRNA in the somite was diversely distributed to the neural bundle near the dorsal vertebral column, spinal nerve, and neuron and myotome development areas. Expression of sFRP2 appears to be associated with myogenesis in chick embryos. In a previous study concerning myogenesis, sFRP2 expression was present in regions of myogenesis in association with Wnt11 expression; sFRP2 may modulate muscle differentiation in these regions [19].

Expression of sFRP2 in the developing spinal cord

Expression of sFRP2 mRNA was detected in the neural tubes, which differentiated into the spinal cord beginning on day 3. The Wnts are known to play essential roles during CNS development [1–3]. Expression of sFRP2 is implicated in modulation of the levels of Wnt signaling, control of proliferation
of the dorsal neural tubes, and development of neural crest precursors [19, 29]. Interestingly, sFRP2 expression was substantially reduced and could only be detected in a small part of the ependyma in the cranial spinal cord on day 9. This suggests that sFRP2 is actively involved in development of the spinal cord until differentiation of the spinal cord is almost complete.

Although the exact role of sFRP2 in embryo development is not fully understood, expression of sFRP2 in multiple organ systems of the chick embryo suggests that the sFRP2 gene plays an active role in development of multiple tissues. Wnt signaling controls development by a variety of mechanisms, such as by modulating cell proliferation, transformation and differentiation [1–4]. Previous studies have proven that sFRP2 is a Wnt antagonist [6, 7, 9]. In the present study, sFRP2 was expressed in a number of developing regions where it may act to modulate the development process during different stages.

Our interest in studying sFRP2 is particularly due to the fact that we found that expression of sFRP2 was absent or minimal in most normal adult canine tissues, but was upregulated in transformed or quickly grown canine malignant tumors [30, 31]. Thus, we believed that sFRP2 must have a role in controlling cell growth or proliferation and analyzed the expression pattern of sFRP2 in chick embryos. It has recently been reported that sFRP2 modulates apoptosis [32–36] and that it is overexpressed in relation to human and canine cancers [30, 31, 37, 38]. This suggests that sFRP2 has important roles not only in embryo development but also in control of hemeostasis of normal tissues. This study provides the basis for further investigation of the mechanism through which development is regulated by sFRP2 in the chick embryo.

References


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