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EXPRESSION OF SCLEROSTIN IN THE DEVELOPING ZEBRAFISH (*DANIO*) BRAIN AND SKELETON

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Abstract

Sclerostin is a highly conserved, secreted, cystine-knot protein which regulates osteoblast function. Humans with mutations in the sclerostin gene (*SOST*), manifest increased axial and appendicular skeletal bone density with attendant complications. In adult bone, sclerostin is expressed in osteocytes and osteoblasts. *Danio rerio* sclerostin-like protein is closely related to sea bass sclerostin, and is related to chicken and mammalian sclerostins. Little is known about the expression of sclerostin in early developing skeletal or extra-skeletal tissues. We assessed *sclerostin* (*sost*) gene expression in developing zebrafish (*Danio rerio*) embryos with whole mount *in situ* hybridization methods. The earliest expression of *sost* RNA was noted during 12 hours post-fertilization (hpf). At 15 hpf, *sost* RNA was detected in the developing nervous system and in Kupffer's vesicle. At 18, 20 and 22 hpf, expression in rhombic lip precursors was seen. By 24 hpf, expression in the upper and lower rhombic lip and developing spinal cord was noted. Expression in the rhombic lip and spinal cord persisted through 28 hpf and then diminished in intensity through 44 hpf. At 28 hpf, *sost* expression was noted in developing pharyngeal cartilage; expression in pharyngeal cartilage increased with time. By 48 hpf, *sost* RNA was clearly detected in the developing pharyngeal arch cartilage. *Sost* RNA was abundantly expressed in the pharyngeal arch cartilage, and in developing pectoral fins, 72, 96 and 120 hpf. Our study is the first detailed analysis of *sost* gene expression in early metazoan development.

Keywords

Sclerostin; *sost*; skeleton; cartilage; brain

1. Introduction

Sclerostin is a secreted glycoprotein that plays an important role in skeletal development and the regulation of osteoblastic activity (Balemans et al., 2001; Balemans and Van Hul, 2004;

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Brunkow et al., 2001; Galus and Wlodarski, 2004; Kusu et al., 2003; Sutherland et al., 2004; van Bezooijen et al., 2004; Winkler et al., 2003). A loss or reduction in expression of the protein, results in the bone dysplasias, sclerosteosis or van Buchem disease (Balemans et al., 2001; Balemans et al., 2002; Brunkow et al., 2001). These diseases are associated with osteoblastic hyperactivity, progressive skeletal overgrowth, defects in craniofacial development and, in sclerosteosis, syndactyly of the second and third digits (Balemans et al., 2001; Balemans et al., 2002; Beighton et al., 1976; Brunkow et al., 2001; Itin et al., 2001; Owen, 1976; Staehling-Hampton et al., 2002; Stein et al., 1983). Affected individuals have distinctive facial features such as asymmetric mandibular hypertrophy, frontal bossing, and midface hypoplasia. As a result of hyperostosis of the skull, cranial nerve foramina are narrowed and impingement of a seventh and eighth cranial nerve occurs, resulting in facial palsy and deafness, respectively. In some individuals with sclerosteosis, intracranial volume is reduced resulting in lethal elevations of intracranial pressure.

Despite knowledge regarding the role of sclerostin in the pathogenesis of sclerosteosis and van Buchem disease, the role of the protein in development has not been carefully assessed. Such experiments could yield valuable information regarding the mechanism of action of sclerostin in the development of structures such as cartilage and bone. The expression of *sost* mRNA has been examined in mouse embryos by *in situ* hybridization (Kusu et al., 2003). The earliest expression of *sost* mRNA was observed on day E14; by day E15, expression on the surfaces of developing mandibular and maxillary bones was seen. In long bones, such as the tibia, *sost* mRNA was mainly detected in the perichondrium of the hypertrophic chondrocyte region and the periosteum of the diaphyseal region at day E16. In the tibia at day E18, *sost* mRNA was detected in the trabecular bone. Strong expression was found in the endothelium of the pharyngeal artery in E11 embryos. In addition, expression was seen in the liver of day E12 embryos, localized to islands of hematopoietic cells.

Bone formation occurs by intra-membranous and endochondral mechanisms which are influenced by a variety of growth factors (Kronenberg, 2003; Reddi, 1994). Since sclerostin influences the activity of the bone morphogenetic proteins (BMP) and wnt signaling pathways, it is likely to play a role in early cartilage development (Ellies et al., 2006; Kamiya et al., 2008; Kusu et al., 2003; Semenov et al., 2005; Semenov and He, 2006; van Bezooijen et al., 2005a; van Bezooijen et al., 2004; van Bezooijen et al., 2007; van Bezooijen et al., 2005b; Winkler et al., 2003; Winkler et al., 2005; Winkler et al., 2004; Yanagita, 2005). It might also be important in the development of other tissues, such as those in the nervous system, in which bone morphogenetic proteins are key players (Aybar et al., 2002; Bainter et al., 2001; Barembaum and Bronner-Fraser, 2005; Barth et al., 1999; Glavic et al., 2004; Golden et al., 1999; Londin et al., 2005; Muroyama et al., 2002; Neave et al., 1997; Tribulo et al., 2003). The developing zebrafish embryo is an ideal model in which to study cartilage and bone formation, because of its transparency, and because cartilage structures in the pharynx and jaw form early in development prior to the onset of ossification. The expression of genes in cartilage can be examined in the absence of osteogenesis which occurs at a later stage. We now show that *sost* RNA and sclerostin protein are detected in pharyngeal and mandibular cartilage of the developing zebrafish. In addition, *sost* RNA is detected early in the developing neural system suggesting a role for this protein in nervous system physiology.

2. Results

2.1 Sclerostin bioinformatics analysis

On searching the zebrafish genome (www.ensembl.org, Zv9, March 2012), one *sost* gene located on chromosome 12 is found. Two *sost-domain containing protein 1a* and *1b* genes are found on chromosome 15 and 19. *Danio* sclerostin protein closely resembles sea bass

sclerostin, chick sclerostin, and to a lesser extent, rat, mouse, hamster, dog, cow, monkey and primate (including human) sclerostins (Figure 1A). A phylogenetic analysis of proteins shows that that *Danio* sclerostin is most closely related to sea bass sclerostin, followed by avian sclerostin, and has more distant relationships to murine and other mammalian sclerostins (Figure 1B).

2.2 Sclerostin mRNA (*sost*) is expressed early in zebrafish development

Expression analysis of *sost* in early zebrafish embryos was conducted using whole mount *in situ* hybridization with sense and anti-sense *sost* probes. Little to no *sost* transcript is observed in zebrafish embryos 4, 6, 8 and 10 hours post-fertilization (hpf) (Figure 2, A, C, E and G, respectively). No signal is observed with the sense *sost* probe at these early time points (Figure 2, B, D, F and H, respectively). The earliest time at which *sost* mRNA is clearly seen is at 12 hpf (Figure 2, I and K, respectively). The signal appears diffuse and is distributed throughout the developing embryo, excepting the yolk. No signal is observed with the sense *sost* probe at this time (Figure 2, J and L). The *in situ* hybridization data for *sost* RNA expression is supported by reverse transcriptase polymerase chain reaction (RT-PCR) with *sost* RNA expression gradually augmenting between 12 – 96 hpf (Figure 2M, right panel labeled “cDNA”). Housekeeping 18S RNA is detected uniformly at all times. Appropriate controls in which reverse transcriptase was omitted from RT-PCR reaction mix showed no bands corresponding to *sost* or 18S (Figure 2M, left panel, labeled “no-RT controls”).

During somitogenesis at 15 hpf, expression of *sost* mRNA is observed in Kupffer’s vesicle (arrow, KV, Figure 3B). As seen at earlier times, no signal is observed in 15 hpf embryos with the sense *sost* probe (Supplemental figure, panel A). At 18 and 20 hpf, *sost* mRNA is clearly seen in the early rhombic lip (Figure 3, D and F, arrow, early rhombic lip or ERL). No signal is observed with the sense *sost* probe (Supplemental figure, panels B, C). Analysis of *sost* mRNA expression at 22 and 24 hpf shows the presence of *sost* RNA in the dorsal anterior spinal cord (SC, arrow, Figure 3, G and I) and in the hindbrain (arrowheads, Figure 3I and J, labeled URL, upper rhombic lip, and LRL, lower rhombic lip). This pattern in the developing nervous system persists through 28 hpf (Figure 4A and B). Between 28 and 44 hpf *sost* mRNA begins to accumulate in the pharyngeal cartilage primordia (arrow PC, lateral view, Figure 4B), with no discernible signal when using the *sost* sense probe (Supplemental figure, panel F). In contrast, the intensity of *sost* hybridization in the nervous system after 28 hpf diminishes in intensity until it is no longer detectable at 44 hpf (Figure 4, E–J).

At 2 and 3 days post-fertilization (dpf) *sost* mRNA expression becomes more apparent and clearly delineated in the developing pharyngeal cartilages (Figure 5, A–F, bracketed area, pharyngeal cartilage, PC). *Sost* RNA is also seen in the developing pectoral fin (Figure 5D–F, pectoral fin, PF). In Figure 6, **panel A**, we show higher power views of hybridization of the anti-sense *sost* probe to a 3 dpf zebrafish larva. Clear hybridization is seen in the ceratobranchial cartilage. These data clearly show intense expression of sclerostin RNA in cartilage, suggesting an important role for sclerostin in cartilage development. No signal is observed with the sense oligonucleotide (Supplemental Figure, panels K, L). Continued and increasing *sost* mRNA expression determined by RT-PCR methods is consistent with the marked expression of this gene in developing cartilage (Figure 2M).

At day 4 and 5 dpf, *sost* mRNA expression becomes well demarcated in the developing pharyngeal cartilages (Figure 5, G–L). At 5 dpf, *sost* mRNA expression in the ceratobranchial and Meckel’s cartilage is apparent (Figure 5, K–L, bracketed area, ceratobranchial, CB; Meckel’s, M). In Figure 6, **panels B and C**, we show higher power views of the hybridization of the anti-sense *sost* probe to a 4 and 5 dpf zebrafish larva. Clear

hybridization is seen in the ceratobranchial 1–5, ceratohyal and Meckel's cartilages. These structures have been confirmed by us (data not shown) and by others (Gavaia et al., 2006; Schilling and Kimmel, 1997; Walker and Kimmel, 2007) to be alcian blue positive (cartilage) and alizarin red negative (bone).

In 5 dpf sclerostin protein is detected in Meckel's, palatoquadrate, basibranchial and ceratohyal cartilages of zebrafish in a punctuate pattern by immuno-histochemistry (data not shown). No immunostaining is seen when pre-immune serum is used.

3. Discussion

The data suggest that maternally derived *sost* mRNA is not present in significant amounts early (<4 hpf) in embryo development, and that transcription of the embryo *sost* gene is required for the accumulation of tissue *sost*. This accrual begins early in zebrafish development, around the 12 hpf mark. Of great interest is the finding that sclerostin expression occurs in the nervous system as early as 15 hours post-fertilization. Sclerostin binds to, and antagonizes the activity of the bone morphogenetic proteins (Kusu et al., 2003; van Bezooijen et al., 2005a; Winkler et al., 2003; Winkler et al., 2004), and because of the critical role of BMPs in the development of the central nervous system in zebrafish (Aybar et al., 2002; Bainter et al., 2001; Barth et al., 1999; Neave et al., 1997; Nguyen et al., 2000; Tribulo et al., 2003), our findings suggest that sclerostin may play a role in modulating signals important for formation of the nervous system in the zebrafish. These findings greatly extend the possible role of sclerostin in developmental biology.

While the function of sclerostin has been primarily assigned to the formation of bone and to the regulation of osteoblast activity, the current data showing expression of *sost* in cartilage tissues suggests an earlier role for this protein in skeletal system development. Several other authors (Du et al., 2001; Gavaia et al., 2006; Schilling and Kimmel, 1997; Walker and Kimmel, 2007) have shown that pharyngeal arch structures (ceratobranchial, ceratohyal, Meckel's and palatoquadrate) are exclusively composed of chondrocytes (alcian blue positive, alizarin red negative) with no calcification from the time of their appearance until >6.5 dpf. At 96 hpf the first calcified pharyngeal teeth appear, attached to ceratobranchial 5 that is still composed of chondrocytes. Markers of bone calcification are absent in the structures we have described until 5–6 dpf (Gavaia et al., 2006). Given the information in the literature, the *sost* positive cells in the pharyngeal arch structures shown are chondrocytes.

The zebrafish is a particularly useful model for studying skeletal formation since cartilage tissues emerge in a temporally distinct manner, at an early time in development. It will be of interest to examine the effects of reducing sclerostin expression in the central nervous system and developing skeleton by morpholino oligonucleotide methods (Ekker, 2000), zinc finger nucleases (Amacher, 2008; Doyon et al., 2008; Foley et al., 2009; McCammon et al., 2011) or TALENS (Huang et al., 2011; Sander et al., 2011). With knock-down of sclerostin expression in the central nervous system, alterations in nervous system development may develop, thus pointing to the importance of sclerostin in central nervous system development. In the skeleton, increases in bone density and alterations in structure or the temporal pattern of appearance of structures may be observed. The zebrafish is an excellent model system in which to sequentially follow such changes.

4. Materials and Methods

4.1 Embryo collection

Zebrafish embryos and larvae were obtained from mating of Segrest wild-type (SWT) parents under controlled barrier conditions and incubated at 28–30°C. Embryos greater than 24 hours post fertilization (hpf) were treated with 0.2 mM PTU (1-phenyl-2-thio-urea). Zebrafish were collected at various stages, fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS: 137 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.2) overnight at 4°C, washed with PBS, dehydrated in ethanol and stored at –20°C until use. Embryos less than 24 hpf were de-chorionated after fixing, prior to storage.

4.2 Riboprobe synthesis

Total RNA was isolated from adult zebrafish using an RNeasy mini kit (Qiagen). RNA (0.4 µg) was used to synthesize cDNA (20 µL) using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The 630 bp coding sequence for zebrafish sclerostin (*sost*) (GenBank XM_001340647) was amplified by PCR using the following primers: 5' primer, 5ZFSOSTBam: GAGAGGATCCATGCAGGTGTCTCTGGCGCTCGTGCCTGCGGC; 3' primer, 3ZFSOSTHind: GAGAAAGCTTGTATGAATTGCTGTTGATGGACGGCACTTTGCTG. The *Bam*HI and *Hind*III restriction enzyme sites, respectively, are in bold. Aliquots (2 µL) of iScript RT reactions were used as template for PCR reactions using Platinum® *Taq* DNA Polymerase, High Fidelity (Invitrogen). After detection of DNA of appropriate mass on agarose gels, the PCR was repeated using 4 µL of the first PCR reaction as template. The PCR product was purified from low melting point agarose using Wizard SV purification columns (Promega). The gel-purified PCR product was cleaved with *Bam*HI and *Hind*III, and ligated into the vector pSP73 cut with *Bam*HI and *Hind*III (Promega). DNA sequence analyses of plasmid preparations from *E. coli* Top10 cells demonstrated the full length coding sequence was identical to XM_001340647 but for 4 nucleotide differences: T192C, A229T, T309C, A524G. This zebrafish Scl-pSP73 construct was used to generate probes for *in situ* hybridization. Briefly, the Scl-pSP73 vector was digested with *Bam*HI (antisense probe) or *Hind*III (sense probe) and purified following gel electrophoresis. Riboprobe *in vitro* transcription system for SP6 and T7 promoters (Promega) was used to generate RNA probes labeled with digoxigenin- rUTP (Roche Diagnostics) according to manufacturer's instructions. Probes were cleaned using RNeasy mini columns (Qiagen) and hydrolyzed at 60°C for 10–12 minutes using an equal volume of probe and of 0.2M Na₂CO₃; pH 10.2. The reaction was stopped by adding 3% vol/vol of 3M sodium acetate, pH 6.0 and 5% vol/vol of 10% glacial acetic acid. Reactions were cleaned using RNeasy mini columns, quantified and verified by gel electrophoresis. Intact probes ran at about 659/680bps (antisense/sense) while hydrolyzed probes appeared at approximately 500bps.

4.3 Whole mount in situ hybridizations

On day 1, embryos were gradually re-hydrated through an ethanol/phosphate buffered saline (PBS) series, washed with PBS + 0.1% Tween 20 (PBST) and digested with 10 µg/ml proteinase K in PBST for periods of time specific to embryo/larval stage (15 seconds to 50 minutes). Embryos were briefly rinsed in PBST, re-fixed in 4% paraformaldehyde (PFA) for 20 minutes and extensively washed in PBST before incubating overnight at 70°C in pre-hybridization buffer (50% de-ionized formamide, 5X SSC, 5 mM EDTA (pH 8.0), 50 µg/ml heparin, 0.5 mg/ml t-RNA, 9.2 mM citric acid, 0.1% Tween-20). On day 2, embryos were placed in pre-hybridization buffer containing 0.25 ng/µl of antisense or sense RNA probe labeled with digoxigenin and incubated overnight at 70°C. The next day, the embryos were washed through a gradient of pre-hybridization wash (PHW: 5X SSC, 50% de-ionized formamide, 5 mM EDTA (pH 8.0), 9.2 mM citric acid, 0.1% Tween-20) and saline-sodium

citrate (SSC – 20X SSC: 3M NaCl, 0.3M Na-citrate, pH 7.0). The first set of washes was performed at 70°C: (i) PHW (quick rinse), (ii) 75% PHW / 25% 2xSSC (15 min), (iii) 50% PHW / 50% 2xSSC (15 min), (iv) 25% PHW / 75% 2xSSC (15 min), (v) 2xSSC (15 min), (vi) 0.2x SSC (30 min, x2 washes). The second set of washes (SSC/PBST series) was performed at room temperature: (i) 75% 0.2x SSC / 25% PBST (5 min), (ii) 50% 0.2x SSC / 50% PBST (5 min), (iii) 25% 0.2x SSC / 75% PBST (5 min), (iv) PBST (5 min). Embryos/larvae were transferred to blocking buffer (2% lamb serum, 2 mg/ml BSA in PBST) and incubated for 4–6 hours at 4°C. During this time, sheep anti-digoxigenin-AP Fab fragments (Roche Diagnostics) were pre-blocked at 4°C with fish acetone powder in blocking buffer. Embryos/larvae were transferred to antibody (1:6,000) in blocking buffer and incubated overnight with gentle agitation at 4°C. The next day, embryos were washed extensively with PBST followed by 3 × 5 min washes with AP buffer (100 mM NaCl, 50 mM MgCl₂, 100 mM Tris (pH 9.5), 0.1% Tween-20). Chromogenic substrates NBT/BCIP (Roche, diluted to 0.225 mg/ml and 0.175 mg/ml, respectively) were added to AP buffer and applied to embryos. Color development proceeded until embryos were washed 3 × 15 min in stop solution (1X PBS, pH 5.5; 1 mM EDTA; 0.1% Tween-20), followed by 1 × 15 minute wash in PBST. Embryos were either mounted into glass capillaries in 75% glycerol in PBST for image capture by SCORE (Petzold *et al.*, 2010), or dehydrated through a PBST/ethanol gradient for storage at –20°C. Composite images of embryos were generated by merging different planes using the software package iSolution.

4.4 RNA extraction and Reverse Transcriptase PCR

Embryos/larvae at various stages of development were collected, flash frozen and stored at –80°C until time of RNA extraction. Total RNA extraction was performed using NucleoSpin® RNA/Protein kit (Macherey-Nagel). Following the manufacturer's instructions, one microgram of total RNA from each isolation was reverse transcribed using the Bio-Rad iScript cDNA Synthesis kit. Primer pair for reverse transcriptase polymerase chain reaction (RT-PCR) amplification of zebrafish sclerostin-like cDNA (*sost*, NCBI acc# XM_001340647.3): Sost122FqPCR (5'-TACCAGAATACGCGGAGGAC-3') and Sost549RqPCR: CGATTGGTTGTGTTGTCGAG. This primer pair spans the first and second exon of *sost*. The housekeeping gene 18S was chosen based on the stable expression pattern of this transcript during zebrafish developmental stages. Primers for 18S were selected based on a previous assessment (McCurley and Callard, 2008). 18sRibQPCR-F (5'-TCGCTAGTTGGCATCGTTTATG-3') and 18sRibQPCR-R (5'-CGGAGGTTCTGAAGACGATCA-3'). Complementary DNA and no-RT samples were diluted in nuclease-free water prior to RT-PCR and amplified for 35 cycles with an annealing temperature of 53°C using HotStarTaq DNA Polymerase (Qiagen), according to manufacturer's instructions. Reactions were supplemented with 5x Q-Solution and 2 mM MgCl₂.

4.5 Generation of anti-sclerostin immune serum and whole mount immunofluorescence

A peptide corresponding to amino acids 188–204 (188-SSKKPRRNKKHRSKVPS-204-C) in zebrafish *sost*, with an extra carboxyl-terminal Cys, was synthesized using solid phase peptide synthetic methods (Stewart and Young, 1984) and conjugated to keyhole limpet hemocyanin in the (MPRC) Mayo Protein Core Facility Peptide Synthesis Facility. Antibodies were raised to the *sost* peptide in rabbits at Cocalico Biologicals using standard methods, and antigen-specific responses were confirmed by a direct ELISA. In addition, the antibodies recognized bacterially produced 1–210 zebrafish *sost*-maltose binding protein (*sost*-MBP) of the appropriate molecular mass, by SDS-PAGE/immunoblot analyses. In order to biosynthesize *sost*-MBP, the *Bam* HI/*Hind* III fragment of zebrafish *sost* in pSP73 (above) was inserted into the *Bam* HI/*Hind* III sites in pMAL-c4E (New England Biolabs). DNA sequence of clones from *E. coli* Top10 cells was the same as the pSP73 construct (4

nucleotide differences (see above) and two coding differences (T77S, H175R) (from XM_001340647). The T77S, H175R 1–210 sost-pMAL-c4E construct was expressed in, and purified from, *E. coli* Origami 2 (DE3) cells (Novagen, EMD) as described (Craig et al., 2010).

Whole mount immunofluorescence was performed using rabbit pre-immune serum and anti-sclerostin immune polyclonal serum, described above. Fixed larvae at 5 dpf were rehydrated through an ethanol/PBST gradient and digested with 10 µg/ml proteinase K for 20 minutes. Larvae were quickly rinsed in water, PBST and re-fixed in 4% PFA in PBS for 20 minutes. Five PBST washes, 5 minutes each, were performed. Larvae were blocked (blocking buffer: 2% normal goat serum, 1% BSA in PBST) for 4 hours at room temperature. Immune or pre-immune sera (1:8000) were applied in blocking buffer and larvae were incubated overnight at 4°C. Following extensive washes (wash buffer: 1% BSA in PBST), Alexa Fluor 555 donkey anti-rabbit IgGs (Invitrogen) were applied in blocking buffer at 2.5 µg/ml. Larvae were incubated in secondary antibodies overnight at 4°C and washed extensively next day in wash buffer. One final wash in PBST was performed before transferring larvae *SlowFade*® Gold antifade reagent (Invitrogen). Fish were mounted into glass capillaries and images were acquired under 85% glycerol by fluorescence microscopy (Petzold et al., 2010).

4.6

Alcian blue and alizarin red staining of zebrafish embryos and larvae This was performed as described by Walker and Kimmel (Walker and Kimmel, 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Sclerostin (*sost*) RNA is expressed early (15 hpf) in *Danio*
- *Sost* expression is seen in the developing brain
- *Sost* is expressed in developing pharyngeal cartilage

Figure 1.

A. Alignment of sclerostins from various species. The consensus sequence is shown on the last line of each block. **B.** Phylogeny of sclerostins.

Figure 2.

Whole mount *in situ* hybridization of early stage zebrafish embryos (4–12 hpf) using anti-sense or sense *Danio sost* probe. Panel A. 4 hpf, anti-sense probe. Panel B. 4 hpf, sense probe. Panel C. 6 hpf, anti-sense probe. Panel D. 6 hpf, sense probe. Panel E. 8 hpf, anti-sense probe. Panel F. 8 hpf, sense probe. Panel G. 10 hpf, anti-sense probe. Panel H. 10 hpf, sense probe. Panel I. 12 hpf, anti-sense probe, lateral view Panel J. 12 hpf, sense probe, lateral view. Panel K. 12 hpf, anti-sense probe, dorsal view. Panel L. 12 hpf, sense probe, dorsal view. Panel M. Reverse transcriptase polymerase chain reaction assessment of *sost* expression and housekeeping gene, 18S. Scale bar = 100 μ m.

Figure 3.

Whole mount *in situ* hybridization of zebrafish embryos (15–24 hpf) using anti-sense *Danio sost* probe. Panel A. 15 hpf, dorsal view. Panel B. 15 hpf, lateral view. Panel C. 18 hpf, dorsal view. Panel D. 18 hpf, lateral view. Panel E. 20 hpf, dorsal view. Panel F. 20 hpf, lateral view. Panel G. 22 hpf, dorsal view. Panel H. 22 hpf, lateral view. Panel I. 24 hpf, dorsal view. Panel J. 24 hpf, lateral view. KV = Kupffer's vesicle; ERL = early rhombic lip; SC = spinal cord; URL = upper rhombic lip; LRL = lower rhombic lip. Scale bar = 100 μ m.

Figure 4.

Whole mount *in situ* hybridization of zebrafish embryos (28–44 hpf) using anti-sense *Danio sost* probe. Panel A. 28 hpf, dorsal view. Panel B. 28 hpf, lateral view. Panel C. 32 hpf, dorsal view. Panel D. 32 hpf, lateral view. Panel E. 36 hpf, dorsal view. Panel F. 36 hpf, lateral view. Panel G. 40 hpf, dorsal view. Panel H. 40 hpf, lateral view. Panel I. 44 hpf, dorsal view. Panel J. 44 hpf, lateral view. SC = spinal cord; URL = upper rhombic lip; LRL = lower rhombic lip; PC = pharyngeal cartilage. Scale bar = 100 μ m.

Figure 5.

Whole mount *in situ* hybridization of zebrafish embryos (2–5 dpf) using anti-sense *Danio sost* probe. Panel A. 2 dpf, dorsal view. Panel B. 2 dpf, lateral view. Panel C. 2 dpf, ventral view. Panel D. 3 dpf, dorsal view. Panel E. 3 dpf, lateral view. Panel F. 3 dpf, ventral view. Panel G. 4 dpf, dorsal view. Panel H. 4 dpf, lateral view. Panel I. 4 dpf, ventral view. Panel J. 5 dpf, dorsal view. Panel K. 5 dpf, lateral view. Panel L. 5 dpf, ventral view. PC = pharyngeal cartilage; PF = pectoral fin; M = Meckel's; CB = ceratobranchial. Scale bar = 100 μ m.

Figure 6. Whole mount *in situ* hybridization of zebrafish embryos (3–5 dpf) using anti-sense *Danio sost* probe. Ventral views. Panel A. 3 dpf. Panel B. 4 dpf. Panel C. 5 dpf. PF = pectoral fin; M = Meckel's; CB = ceratobranchial. CH = ceratohyal. Scale bar = 200 μ m.