A pair of Sox: distinct and overlapping functions of zebrafish sox9 co-orthologs in craniofacial and pectoral fin development

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Summary

Understanding how developmental systems evolve after genome amplification is important for discerning the origins of vertebrate novelties, including neural crest, placodes, cartilage and bone. Sox9 is important for the development of these features, and zebrafish has two co-orthologs of tetrapod SOX9 stemming from an ancient genome duplication event in the lineage of ray-finned fish. We have used a genotype-driven screen to isolate a mutation deleting sox9b function, and investigated its phenotype and genetic interactions with a sox9a null mutation. Analysis of mutant phenotypes strongly supports the interpretation that ancestral gene functions partitioned spatially and temporally between Sox9 co-orthologs. Distinct subsets of the craniofacial skeleton, otic placode and pectoral appendage express each gene, and are defective in each single mutant. The double mutant phenotype is additive or synergistic. Ears are somewhat reduced in each single mutant. The double mutant is additive or synergistic. Ears are somewhat reduced in each single mutant but are mostly absent in the double mutant. Loss-of-function animals from mutations and morpholino injections, and gain-of-function animals injected with sox9a and sox9b mRNAs showed that sox9 helps regulate other early crest genes, including foxd3, sox10, snai1b and crestin, as well as the cartilage gene co12a1 and the bone gene runx2a; however, tfap2a was nearly unchanged in mutants. Chondrocytes failed to stack in sox9a mutants, failed to attain proper numbers in sox9b mutants and failed in both morphogenetic processes in double mutants. Pleiotropy can cause mutations in single copy tetrapod genes, such as Sox9, to block development early and obscure later gene functions. By contrast, subfunction partitioning between zebrafish co-orthologs of tetrapod genes, such as sox9a and sox9b, can relax pleiotropy and reveal both early and late developmental gene functions.

Key words: Chondrogenesis, Craniofacial, Gene duplication, Genome duplication, Limb morphogenesis, Skeletogenesis, Subfunction partitioning

Introduction

Conserved genetic pathways control development of chordate features (e.g. Bassham and Postlethwait, 2000; Satoh, 2003; Seo et al., 2004; Yasuo and Satoh, 1998). To shared chordate characters, vertebrates appended evolutionary novelties, including paired sense organs, cartilage, and bone (Gans and Northcutt, 1983; Northcutt and Gans, 1983; Shimeld and Holland, 2000). Several of these features derive from two tissues thought to be vertebrate innovations: neural crest, which gives rise to pigment cells, peripheral neurons, glia, craniofacial cartilage, and bone (e.g. Eisen and Weston, 1993), and epidermal placodes, which form ears, olfactory organs, lens, lateral line, and some cranial ganglia (Begbie and Graham, 2001). The evolutionary origin of developmental mechanisms leading to neural crest and placodes is not yet fully understood (Begbie and Graham, 2001; Graham and Begbie, 2000; Graham et al., 2004; Jeffery et al., 2004; Meulemans et al., 2003; Shimeld and Holland, 2000).

The transcription factor Sox9 functions in the development of crest, placodes, cartilage, and bone. Sox9 promotes crest-like behaviors in neural plate cells and biases cells towards glial and melanocyte fates (Cheung and Briscoe, 2003). Sox9 also helps determine crest-derived chondrogenic lineages (Mori-Akiyama et al., 2003; Spokony et al., 2002; Yan et al., 2002), and it functions in morphogenesis and differentiation of cartilage and bone (Bi et al., 1999; Bi et al., 2001; Yan et al., 2002; Zelzer and Olsen, 2003). Despite these advances, the mechanisms by which Sox9 acts in crest development are incompletely understood. Sox9 also functions in placode development, including the otic placode (Liu et al., 2003; Saint-Germain et al., 2004), and human patients with mutations in Sox9 sometimes lack olfactory bulbs (Houston et al., 1983). Given its role in crest and placode development, elucidating the mechanisms of Sox9 action could help us understand the origin of vertebrate developmental innovations.

Our investigation of Sox9 function exploits a special situation in teleost fish, the possession of two co-orthologs of tetrapod Sox9 (Chiang et al., 2001; Cresko et al., 2003; Koopman et al., 2004; Li et al., 2002). These duplicated genes arose in an ancient genome duplication event that preceded the
teleost radiation (Amores et al., 1998; Koopman et al., 2004; Meyer and Scharlt, 1999; Postlethwait et al., 1998; Postlethwait et al., 2000; Postlethwait et al., 2002; Taylor et al., 2003; Vogel, 1998; Wittbrodt et al., 1998). Although both genes still bind Sox9-binding enhancer sequences in DNA (Bell et al., 1997; Chiang et al., 2001; Ng et al., 1997), gene expression studies suggest that ancestral Sox9 gene subfunctions partitioned between the two duplicates, leaving each with a subset of the original gene’s functions (Chiang et al., 2001; Cresko et al., 2003; Li et al., 2002; Liu et al., 2003; Yan et al., 2002). This behavior is predicted to be frequent in the evolution of duplicated genes (Force et al., 1999; Hughes, Yan et al., 2002). Subfunction partitioning in teleosts may provide advantages for analysis (Postlethwait et al., 2004), and haploinsufficiency of Sox9 mutations in mammals makes it difficult to obtain homozygous embryos for investigation (Bi et al., 2001; Mori-Akiyama et al., 2003; Sock et al., 2003); but we found that heterozygotes for mutations in zebrafish sox9 co-orthologs are viable and produce homozygous mutant embryos.

To investigate the roles of Sox9 in the development of neural crest and placodes, we conducted a genotype-driven screen for a mutation that deletes sox9b activity. We studied the phenotype of sox9b mutants, and compared them in single- and double-mutant combinations with jellyfish (jef+) (Yan et al., 2002), a null mutation in sox9a (Yan et al., 2002). Results reveal distinct roles for sox9a and sox9b in development of crest, otic placode, cartilage, and bone, and illustrate how subfunction partitioning between teleost co-orthologs of human genes can facilitate the analysis of conserved gene function. Finally, we suggest that subfunction partitioning after duplications producing Sox8, Sox9 and Sox10 may have facilitated the evolutionary origin of neural crest and placodes.

Materials and methods

Genomic clones of sox9b (submitted as AY627769) were isolated by PCR from genomic DNA of 3 dpf (days post fertilization) zebrafish embryos using primers sox9b+296 CACCGGGACGAGCAAGGT and sox9b-644 GTCTGGCTGTATTTTGATGTCG-GATG obtained from the cDNA sequence AF277097, which amplify most of the 5′ end of the gene, and primers sox9b+555 AGGGCGAGAAGCGTCCGTTTGT and sox9b-1695 AGCGCAC-TGCAGATTGGA, which amplify most of the 3′ end. Morpholino antisense oligonucleotides (MO) for sox9b (Gene Tools, Philomath, OR) were: intron 2 splice donor junction (e2i2) TGCACTAATTTACCGAGTGTCTC, and intron 2 splice receptor junction (i2e3) GCCCTGAGACTGACCTGACACACA. A mixture of both MOs (1 ng each) was injected into one-cell stage embryos. MOs for sox9a were as described (Yan et al., 2002).

In situ hybridization was performed as described (Jowett and Yan, 1996). Alcian blue and Alizarin red stained cartilage and bone in fixed larvae as described (Kimmel et al., 1998; Kimmel et al., 2003). Confocal imaging of live embryos and TUNEL assay was performed as described (Crump et al., 2004; Gavrieli et al., 1992).

The sox9b KO/− mutation was made according to Fritz et al. (Fritz et al., 1996). Developing embryos were subjected to 150 to 300 rads of gamma radiation from a cesium-137 source at 2 hpf (hours post fertilization). 126 G0 females were squeezed to make G1 haploid embryos (Streisinger et al., 1981), of which 12 from each female were screened by PCR for loss of the sox9b 3′ UTR using primers sox9b+1461 CTCCTGCCCCTACATCCACTCTC and sox9b-1695 AGCGCACTGCAAGTTGGA. To control for sample loading, we simultaneously amplified the 3′UTR of dlc (NM_130944) using primers dlcF-GAGACTTGAGACCGGAAC- GAAC and dlcR-AATAAAAGGCAAATACCTCCACAG. From one G0 female, six of twelve haploids lacked the sox9b but not the dlc amplicon, and the mutation was inherited in Mendelian fashion. Deficiency mapping was done by PCR on DNA of haploid embryos from carrier females using Z-markers, gene-specific, and contig end-specific primers. To identify b971 homozygotes, we amplified sox9b with dlc as internal control, and jef+1134 (sox9b+) heterozygotes were identified by PCR as described (Yan et al., 2002).

To make mRNA for rescue and ectopic expression experiments, we inserted sox9a and sox9b cDNA into pSP64T (Chiang et al., 2001; Krieg and Melton, 1984). The plasmid DNAs were linearized with BamHI, and were transcribed in vitro with SP6 RNA polymerase using the SP6 mMESSAGE mMACHINE kit (Ambion). For mRNA injections, 50 pg mRNA was injected into 1-4-cell stage zebrafish embryos.

Results

sox9b co-orthologs are expressed in shared and gene-specific domains

To interpret mutant phenotypes, we must first understand gene expression. The ovary provides sox9b as a maternal message that disappears during cleavage in zebrafish and Xenopus (Chiang et al., 2001; Li et al., 2002; Spokony et al., 2002). In 14 hpf embryos, the expression level of sox9a was high in the otic placode and somites (Fig. 1A). In contrast, the expression level for sox9b was high in the developing ear, eye, pre-chordal plate, and cranial and trunk premigratory neural crest (Fig. 1B). Each gene has specific expression domains, for example, sox9b is expressed at high levels in the cranial and trunk crest, and reciprocally, sox9a is expressed at high levels in the somites. Cross-sections anterior to the otic vesicle show that sox9b is expressed at higher levels than sox9a in cranial crest (Fig. 1C,F) (Li et al., 2002). Expression of sox9b downregulates in the crest shortly after cells begin to migrate.

By 26 hpf, sox9a expresses at higher levels than sox9b in the pharyngeal arches (Fig. 1D). In contrast, sox9b expresses in the developing eye and in a dorsal-ventral band in each hindbrain rhombomere (Fig. 1E). At 48 hpf (Fig. 1G,H), both genes are expressed in similar domains in the hindbrain, whereas the tectum and eyes express sox9b, but not sox9a (Chiang et al., 2001).

Pectoral fins and pharyngeal arches express both sox9 genes (Chiang et al., 2001), but in different domains. In the pectoral fin bud at 48 and 68 hpf (Fig. 1G-J), precursors of the scapulocoracoid express sox9a but not sox9b. In contrast, the endochondral disc expresses sox9b at high levels, especially distally, but expresses sox9a at lower levels. In the arches, sox9a expresses at high levels in cartilage precursors deep in the arch and at lower levels in a surrounding single cell layer, the perichordium (Fig. 1K,L), which later makes osteoblasts and forms a bone collar around the cartilage (Caplan and Pechak, 1987). In contrast, sox9b expresses in the ectoderm and endoderm of the epithelial sheath that surrounds the sox9a-expressing perichordium and cartilage (Fig. 1M,N).

This analysis and earlier results (Chiang et al., 2001; Li et al., 2002; Liu et al., 2003; Yan et al., 2002), show that sox9a and sox9b transcripts accumulate in overlapping and unique domains. Gene expression, however, does not equate to gene function. The jef mutation destroys sox9a activity (Yan et al.,
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but to learn the role of sox9b, we mounted a genotype-driven screen.

b971 deletes sox9b

Using our previous strategy (Fritz et al., 1996), we conducted a genotype-driven screen for mutation of sox9b. Among 252 gamma ray-treated chromosomes, one lacked the sox9b PCR amplification product. This mutation, called b971, is inherited as a simple Mendelian recessive, and mapped to the lower end of LG3 (data not shown), the site of sox9b (Chiang et al., 2001). Haploid b971 embryos were screened by PCR for loci previously mapped near sox9b (Barbazuk et al., 2000; Geisler et al., 1999; Hukriede et al., 1999; Knapik et al., 1998; Postlethwait et al., 2000; Postlethwait et al., 1998; Woods et al., 2000). Results showed that b971 is a terminal deletion removing about 10 cM of the lower tip of LG3 (Fig. 2A).

Embryos homozygous for sox9b<sup>b971</sup> do not contain sox9b transcript (Fig. 2B,C), as expected for a deletion. The mutation also deletes an EST for sox8 (fc23c10) (Cresko et al., 2003), but because it is not expressed at least up to hatching (data not shown), it is unlikely to affect the phenotype of b971 embryos.

To control for other ESTs deleted in b971, we treated wild types with sox9b morpholinos as explained below. Note that the splice-directed MOs caused sox9b transcript to accumulate in the nucleus rather than in the cytoplasm (Fig. 2D,E), suggesting that it remained in an unspliced form, as observed previously for sox9a (Yan et al., 2002), and confirming MO efficacy.

Cartilage phenotypes in Sox9-depleted animals

To understand sox9b function, we examined mutant phenotypes. Homozygous sox9b<sup>b971</sup> embryos and double sox9

Fig. 1. Embryonic expression patterns. A,C,D,G,I,K,L, sox9a; B,E,F,H,J,M,N, sox9b. (A,B) 14 hpf, dorsal view. (C,F) Cross-sections of 14 hpf embryos at anterior-posterior level indicated by black bars in A and B. (D,E) Lateral views of 26 hpf embryos. (G,H) Lateral view, 48 hpf. (I,J) Pectoral fin bud at 68 hpf. (K-N) High (K,N) and low (L,M) magnification of sections of 68 hpf embryos through the arches. Abbreviations: co, scapulocoracoid; f, forebrain; fb, fin bud; e, eye; ed, endochondral disc; hb, hindbrain; m, midbrain; mhb, midbrain-hindbrain boundary; nc, neural crest; ov, otic vesicle; pa, pharyngeal arches; pcp, prechordal plate; r, rhombomeres; s, somites; t, tectum. Scale bars: in N, 50 µm for K,N; in B, 100 µm for A,B; in F, 100 µm for C-F; in H, 100 µm for G,H; in J, 100 µm for I,J; in M, 100 µm for L,M; in N, 100 µm for K,N.

Fig. 2. sox9b<sup>b971</sup> linkage map and sox9b expression, and sox9b morpholino-treated embryos. (A) LG3 (distance in cM), with red markers shown to be deleted, blue markers shown not to be deleted, and black markers untested. Region deleted, pale red. (B,C) Lateral views of 24 hpf embryos probed for sox9b expression. (B) wild type; (C) sox9b<sup>b971</sup>. Mutant embryos lack sox9b expression. (D,E) Dorsal, right side view of cranial crest in four-somite stage embryos probed for sox9b expression. (D) Wild type; (E) embryo injected with sox9b splice-directed MO showing retention of sox9b transcript in the nuclei. Scale bars: in C, 100 µm for B,C; in E, 100 µm for D,E.
mutants do not straighten their body axis as wild types and sox9ahi1134 mutants do, giving a curly-down body axis (Fig. 3A,C,E). This phenotype could result from the brief expression of sox9b in the notochord at about 20 hpf. Animals injected with sox9b MOs also show the curly-down phenotype, supporting this hypothesis (Fig. 3D).

To understand the role of sox9b in cartilage development, we stained mutants with Alcian blue. Results showed that homozygous sox9b b971 larvae have reduced first and second pharyngeal arches and neurocranial cartilages (Fig. 3F,H). Ventral views show that sox9ahi1134 animals retain only a few cells of the ceratohyal (Fig. 3K,L), but sox9b b971 larvae preserve portions of several cartilages (Fig. 3M). This shows that sox9a plays a more important role than sox9b in the development of many pharyngeal cartilages. Although both single mutants retain portions of some pharyngeal cartilages, double mutants lack all pharyngeal cartilages (Fig. 3J,O). We conclude that sox9a and sox9b have both redundant and gene-specific functions in pharyngeal cartilage development.

The neurocranium responds like the arches. Mutations in sox9a delete all neurocranial cartilages except a few unidentified nodules (Fig. 3P,Q). In contrast, in sox9b b971 mutants, the neurocranium, including rostral ethmoid plate, anterior basicranial commissure cartilages, posterior basicapsular commissures, and occipital arches, is merely reduced (Fig. 3R). Animals injected with sox9b MO show a similar phenotype (Fig. 3I,S). Double mutants lack all traces of the neurocranium (Fig. 3T).

The sox9 genes also have overlapping and distinct roles in the pectoral fin. The wild-type pectoral appendage includes cleithrum (dermal bone), scapulocoracoid (chondral bone), endochondral disc (or endoskeletal disc), and dermally derived actinotrichia (Grandel and Schulte-Merker, 1998) (Fig. 3U). Lack of sox9a function deletes the scapulocoracoid, but not the cleithrum, endochondral disc, or actinotrichia (Fig. 3V). As with sox9a, lack of sox9b leaves the cleithrum unchanged, but also leaves the scapulocoracoid intact, while decreasing the size of the endochondral disc and the number of actinotrichia, although those remaining are of normal length (Fig. 3W). Animals treated with sox9b MO have the same defect (Fig. 3X), showing that this mutant phenotype is due to lack of sox9b function. In the double mutant, the scapulocoracoid, endochondral disc, and nearly all of the actinotrichia disappear, leaving only the cleithrum (Fig. 3Y). The phenotype of the double mutant is more extreme than the sum of the phenotypes of the single mutants: the endochondral disc and most actinotrichia are missing from the double mutant.

**Fig. 3.** Wild-type, sox9 mutants, and sox9b-morpholino-treated animals. (A-E) Live larvae; (F-J) lateral view of head of Alcian-stained larvae. (K-O) Flat mount of Alcian-stained pharyngeal arches. (P-T) Flat mount of Alcian-stained neurocranium. (U-Y) Alcian-stained pectoral fin bud. All animals at 4 dpf. (A,F,K,P,U) Wild type; (B,G,L,Q,V) sox9a mutant, showing deletion of most cranial cartilage and scapulocoracoid; (C,H,M,R,W) sox9b b971; neurocranium, arches, and endochondral disc reduced; (D,I,N,S,X) sox9b MO-injected animals phenotypically similar to sox9b b971; (E,J,O,T,Y) double mutant, arches, neurocranium, and fin deleted. Abbreviations: abc, anterior basicranial commissure; cbs, ceratobranchials; ch, ceratohyal; m, Meckel’s cartilage; cl, cleithrum; co, scapulocoracoid; ed, endochondral disc; ep, ethmoid plate; hs, hyosymplectic; j, jaw; ov, otic vesicles; no, notochord; pc, parachordal; pq, palatoquadrate; tr, trabecula. Scale bars: in E, 100 µm for A-E; in T, 100 µm for F-T; in Y, 100 µm for U-Y.
Because fin bud development was substantially altered in sox9 mutants, we wondered whether the conserved genetic pathway that controls appendage patterning (Capdevila and Izpisua Belmonte, 2001; Richardson et al., 2004; Tanaka et al., 2000) was intact in double mutants. We therefore examined the expression patterns of two components, shh, which is expressed in the zone of polarizing activity (Neumann et al., 1999), and dlx2a, which is expressed in the apical ectodermal ridge (Akimenko et al., 1994). In the pectoral fin buds of 34 and 52 hpf embryos, expression of both genes was highly similar in normal animals, single mutants, and double sox9 mutants (Fig. 4). We conclude that sox9 activity, although essential for the development of the cartilaginous components of the proximal fin bud, is not required for the establishment of the main signaling centers in appendage development.

Can wild-type sox9b mRNA rescue sox9b<sup>b971</sup> animals?

Morpholino experiments supported the hypothesis that phenotypes observed in sox9b<sup>b971</sup> animals are due to the lack of sox9b function. Because the sox9b<sup>b971</sup> mutation deletes several ESTs (Fig. 2A), however, we wanted to confirm that phenotypes we observed are due to deletion of sox9b. To test whether wild-type sox9b activity could rescue sox9b<sup>b971</sup> phenotypes, we injected in vitro-synthesized wild-type sox9b mRNA into one- to four-cell stage homozygous sox9b<sup>b971</sup> embryos and their phenotypically wild-type siblings. Despite the mosaic distribution of injected transcripts (Ekker, 1999), and the late phenotype we are examining, which results in mRNA dilution and gives opportunities for mRNA degradation, sox9b mRNA caused substantial rescue of Alcian stained pharyngeal cartilages (Fig. 5A,C,E,G,I) and rostral extension of the ethmoid plate in the neurocranium (Fig. 5B,D,F,H,J). We conclude that the cartilage phenotypes observed in the craniofacial skeleton of homozygous sox9b<sup>b971</sup> animals result from loss of sox9b function.

sox9 mutations and bone development

To understand the role of sox9 in craniofacial bone development, we studied runx2b in sox9 mutants. In mammals, RUNX2 acts as the master regulatory gene for osteoblastogenesis (Karsenty and Wagner, 2002). Wild-type embryos at 2 dpf (Fig. 6A) express runx2b in precursors of the bony skeleton (Flores et al., 2004). This pattern is nearly normal in sox9ahi1134 mutants (Fig. 6B), but in sox9b<sup>b971</sup> mutants, in wild-type animals injected with sox9b MOs, and in double mutants, runx2b expression is substantially reduced or missing from pharyngeal arches (Fig. 6C-E).

To directly study bone development, we stained living larvae with Alizarin at 5-7 dpf, a stage at which many bones have begun to differentiate in wild types (Fig. 6F). In sox9ahi1134 mutants (Fig. 6G), some dermal bones were somewhat smaller than normal (dentary, opercular, maxillary), whereas others
were relatively unaffected (cleithrum). In contrast, many cartilage replacement bones were completely missing (ceratohyal, and hyomandibular), and others were much smaller than normal (ceratobranchial-5) in sox9b\textsuperscript{bb971} mutants. In sox9b\textsuperscript{bb971} and animals treated with sox9b MOs (Fig. 6H,I), however, all cartilage-replacement bones and most dermal bones were missing, with the exception of the cleithrum and the opercle, which were reduced. Double mutants had bone defects similar to sox9b\textsuperscript{bb971} single mutants (Fig. 6J), suggesting that the residual dermal bones remaining in sox9b\textsuperscript{bb971} mutants are not due to redundancy with sox9a. We conclude that sox9a is more important for the formation of cartilage-replacement bones, and sox9b is important in the formation of both cartilage-replacement and dermal bones in the cephalic and pectoral fin skeletons.

sox9 mutations and pigment cell development

In addition to craniofacial cartilages, neural crest forms melanocytes, xanthophores, and iridophores (Rawls et al., 2001). A striking feature of sox9b\textsuperscript{bb971} homozygotes and double mutants is the presence of melanocytes with dispersed melanosomes even in the light (Fig. 3C; Fig. 7A,C,D). To learn whether sox9b affects melanocyte development, we examined expression of dopachrome tautomerase (dct), an enzyme of melanin biosynthesis (Kelsh et al., 2000), and found that the number, size, and distribution of dct-expressing cells was normal in sox9b\textsuperscript{bb971} animals (Fig. 7E-H). This suggests that the melanocyte phenotype may be a function of cell physiology rather than development. Xanthophores appeared normal in sox9b\textsuperscript{bb71} animals, as did the distribution of cells expressing xanthine dehydrogenase mRNA (data not shown). Dark field microscopy showed that sox9a-MO mutants have the normal number and distribution of iridophores (Fig. 7J-L), but sox9b\textsuperscript{bb71} and double mutants had reduced populations of iridophores (Fig. 7K,L). Melanocyte and iridophore phenotypes were like sox9b\textsuperscript{bb71} mutants in animals injected with sox9b-MO (Fig. 7C,K; data not shown). We conclude that sox9b is essential for normal development of iridophores and for the distribution of melanosomes within melanocytes.

The roles of sox9 in otic vesicle development

Mutant phenotypes showed that sox9 co-orthologs are important for development of the ear, a derivative of the otic placode. In sox9a\textsuperscript{ahi1134} mutants, the otic vesicle and otoliths are nearly normal (Fig. 7M,N) (Liu et al., 2003; Yan et al., 2002). In sox9b\textsuperscript{bb971} mutants, the ear is smaller than normal and forms small, but otherwise normal otoliths (Fig. 7O), confirming our previous analysis using sox9b MOs (Liu et al., 2003). The otic vesicle in double mutants, however, was usually missing, or if present, consisted of vestiges lacking normal otoliths (Fig. 7P). Antibody to Pax2, an otic placode marker (Puschel et al., 1992), failed to label the otic region in double mutants (data not shown), suggesting that the otic placode does not form in sox9-deficient zebrafish. Alizarin staining confirmed the absence of otoliths in double mutants (Fig. 6J). The enhanced ear phenotype of the double mutant compared to either single mutant suggests partially overlapping functions of sox9 co-orthologs in otic placode development.

What is the position of sox9 in the hierarchy of crest gene expression?

Our in situ hybridization experiments (Fig. 1B) showed that sox9b is expressed at high levels in premigratory neural crest about as early as other early crest markers such as foxd3 (formerly fkd6) (Odenthal and Nusslein-Volhard, 1998), snail1b (formerly snail2) (Thissie et al., 1995), and tfap2a (formerly AP2alpha) (Barrallo-Gimeno et al., 2004; Knight et al., 2003; Knight et al., 2004; O’Brien et al., 2004). Double in situ hybridization experiments with sox9b and either foxd3 or snail1b showed that these three genes are expressed in largely overlapping cell populations in this domain (data not shown).

Results showed that at the three-somite stage, sox9a\textsuperscript{ahi1134} embryos express snail1b nearly normally (Fig. 8A,B), but sox9b\textsuperscript{bb71} embryos have a substantially smaller snail1b expression domain (Fig. 8C). The same results were obtained with foxd3 and sox10 (data not shown). We used three different combinations of mutations and MOs to generate animals lacking activity of both sox9a and sox9b (sox9b\textsuperscript{bb971};sox9a-MO and sox9b-MO;sox9a\textsuperscript{ahi1134}, and sox9b\textsuperscript{bb971};sox9b\textsuperscript{bb1134} double mutants). All combinations showed greatly reduced expression of snail1b (Fig. 8D shows only the double mutant, and results
with foxd3 and sox10 expression were similar to snail1b at the three-somite stage, data not shown). We conclude that sox9b function is required for full development of the snail1b-, foxd3- and sox10-expressing neural crest subpopulation in three-somite stage embryos.

The transcription factor tfap2a is expressed early in crest development in zebrafish and other vertebrates (Barrallo-Gimeno et al., 2004; Knight et al., 2004; Knight et al., 2003; Luo et al., 2003; O’Brien et al., 2004) (http://zfin.org). We examined tfap2a expression in zebrafish embryos homozygous for sox9a or sox9b mutations either singly or in double mutant combinations, and found no difference from wild type (data not shown), suggesting that sox9 genes are not upstream of tfap2a in zebrafish.

To learn whether sox9 genes play a role in migrating neural crest, we examined dlx2a, which is expressed in a subset of premigratory, and postmigratory crest that forms pharyngeal arches (Akimenko et al., 1994; Kelsh and Raible, 2002; Miller et al., 2000). Results showed that dlx2a-expressing cells were unaffected in sox9ahi1134 embryos (Fig. 8E,F), even though sox9a and dlx2a are expressed in very similar domains. In contrast, the dlx2a-expression domain was somewhat smaller in sox9bb971 embryos and double mutants (Fig. 8G,H). This shows that sox9 function is not essential for cranial crest migration in zebrafish, but that sox9b may help determine the size of the dlx2-expressing cell population.

To discover the effect of sox9 on chondrocyte differentiation, we investigated col2a1 (Yan et al., 1995), which encodes a major collagen of cartilage. Embryos homozygous for sox9ahi1134 or wild-type embryos injected with sox9a MO (data not shown) had, by 68 hpf, little expression in the pharyngeal arches, otic vesicle, and eye capsule (Fig. 8I,J). In contrast, sox9bb971 homozygotes (Fig. 8K) or wild-type
embryos injected with sox9b MO (data not shown) had much smaller col2a1 expression domains in the arches. In the pectoral fin, sox9a mutants lacked col2a1 expression in the scapulocoracoid and had reduced expression in the endochondral disc, whereas sox9b mutants had a smaller col2a1 domain in the endochondral disc but a normal scapulocoracoid domain. In the double mutant (Fig. 8L) and in sox9ahi1134 embryos injected with sox9b MO (data not shown), col2a1 expression was greatly reduced in the arches and ear, was absent from the scapulocoracoid region, and was reduced in the endochondral disc. We conclude that sox9a and sox9b are required either for the expression of col2a1 in overlapping sets of cartilaginous cells, or for the survival and/or proliferation of cells that normally express col2a1.

Colorless (sox10) is expressed early in most premigratory neural crest, a subpopulation of trunk crest cells migrating in the medial pathway (Dutton et al., 2001), and in craniofacial ectomesenchyme (Kelsh and Raible, 2002). Expression of sox10 was nearly normal in trunk crest of homozygous sox9ahi1134 embryos (Fig. 8M,N,Q,R). In homozygous sox9b977 (Fig. 8O,S), however, and in animals abrogated for function of both sox9 co-orthologs (double mutants (Fig. 8P,T); sox9b977;sox9a-MO, and sox9b-MO;sox9ahi1134 (data not shown)), the extent of the sox10 expression domain in the trunk was reduced, and sox10-expressing cells did not migrate as far ventrally as normal. Results for crestin (Luo et al., 2001; Rubenstein et al., 2000) were similar (data not shown). We conclude that sox9b and to a lesser extent, sox9a, is required for full development of sox10- and crestin-expressing neural crest.

To complement these loss-of-function experiments, we conducted gain-of-function experiments to investigate regulatory interactions among early crest genes. In wild-type embryos injected with sox9a or sox9b mRNAs, the injected mRNA was mosaically distributed in a portion of each embryo (compare Fig. 9A and B, and Fig. 9D and F). Injection of sox9a mRNA caused ectopic expression of sox9b (Fig. 9E), and vice versa (Fig. 9C). This suggests that sox9a and sox9b have a mutually positive regulatory influence on each other, confirming our earlier results for ear development (Liu et al., 2003; Hans et al., 2004). Injection of sox9 mRNAs into zebrafish early cleavage stage embryos showed that both sox9a and sox9b caused ectopic expression or rostral extension of the expression domains for foxd3, sox10 and snai1b (Fig. 9 G-O). We conclude that foxd3, sox10 and snai1b can be upregulated by sox9 genes in crest development, consistent with the results from the loss-of-function experiments.

The roles of sox9 genes in cartilage morphogenesis

In living 48 hpf Bodipy ceramide-stained embryos imaged by confocal microscopy, we could not unambiguously distinguish wild-type from mutant embryos (Fig. 10A-D'). By 72 hpf, chondrocytes were stacking in orderly rows in wild-type embryos, but in sox9ahi1134 mutants, chondrocytes failed to stack (Fig. 10E-F'). Mutants homozygous for sox9b977 had fewer chondrocytes, which, however, did stack (Fig. 10G,G'). Double mutants showed fewer chondrocytes and no stacking (Fig. 10H,H'). We conclude that sox9a and sox9b play different roles in cartilage morphogenesis, with sox9a necessary for stacking and sox9b essential for attaining the proper number of chondrocytes.

Small cartilages in sox9b977 mutants could be due to slow cell proliferation rates or elevated death of cartilage progenitor cells, among other possibilities. To test the cell death hypothesis, we performed TUNEL assays on various genotypes at 24 hpf. The results showed (Fig. 11) that homozygotes for sox9b977 or double mutants or animals injected with sox9b-MO had increased TUNEL staining in the central nervous system and pharyngeal cartilage precursors (Fig. 11C-E). This result shows that sox9b helps prevent cell death but sox9a does not. In mouse limb buds, Sox9 also suppresses apoptosis (Akiyama et al., 2002). This result does not rule out the possibility that cell proliferation may also be diminished in sox9b mutants.

**Discussion**

How do developmental mechanisms evolve after genome duplication? This question is important for understanding the origin of vertebrates, because genome duplication may have preceded the evolution of vertebrate features such as neural crest and placodes (Force et al., 2002; Hokamp et al., 2003; Holland et al., 1994; Hughes, 1999; Larhammar et al., 2002; Ohno, 1970; Sidow, 1996; Skrabanek and Wolffe, 1998). Furthermore, related human diseases are sometimes caused by mutations in paralogous genes that arose in ancient genome amplifications, such as campomelic dysplasia (SOX9) and
Waardenburg syndrome (SOX10), or osteogenesis imperfecta (COL1A1) and spondyloepiphyseal dysplasia (COL2A1). The general principles underlying these mechanisms will become evident by investigating mutations in pairs of gene duplicates.

The discovery of a genome duplication in zebrafish ancestry (Amores et al., 1998; Postlethwait et al., 1998), and the revelation that it may have occurred before the teleost radiation (Amores et al., 1998; Koopman et al., 2004; Meyer and Schartl, 1999; Postlethwait et al., 1998; Postlethwait et al., 2000; Postlethwait et al., 2002; Taylor et al., 2003; Vogel, 1998; Wittbrodt et al., 1998) provides opportunities to learn how developmental pathways evolve after all genes of the pathway are duplicated. In the parallel model, duplicated genes sort into two parallel but non-interacting pathways, for example different duplicates act exclusively in different cell types. Under the network model, a reticulated pathway of regulation would interweave the functions of gene duplicates at several levels of the pathway.

In mammals, Bmp2, Msx2, Sox9, Col2a1, Runx2 and Col1a1 interact in the developmental pathway leading from neural crest to cartilage and bone (Healy et al., 1999; Mori-Akiyama et al., 2003; Takahashi et al., 2001). Zebrafish has duplicate copies of these six genes (Chiang et al., 2001; Ekker et al., 1997; Fisher et al., 2003; Flores et al., 2004; Martinez-Barbera et al., 1997; Postlethwait, 2004; Yan, et al., 1995; Yan et al., 2002), and we have begun to investigate their regulatory interactions to test the parallel and network models for the evolution of developmental pathways after genome duplication. In experiments reported here, we made null-activity animals for one of the two Sox9 co-orthologs, and compared them to mutants for the other Sox9 co-ortholog. The network model appears to receive initial support because under the network model of regulation, a reticulated pathway of regulation would interweave the functions of gene duplicates at several levels of the pathway.

**Sox9 expression patterns and subfunction partitioning**

Our side-by-side comparison of Sox9a and Sox9b expression patterns extends earlier reports (Chiang et al., 2001; Li et al., 2002; Liu et al., 2003; Yan, et al., 2002), and shows that even when Sox9a and Sox9b are expressed in the same organs, the precise cell type or timing can be different. For example, in the pectoral appendage, Sox9a is expressed at high levels in the girdle and at low levels in the endochondral disc, while reciprocally, Sox9b is not expressed in the girdle but is expressed at high levels in the endochondral disc. In mouse, Sox9 is expressed in both the limb girdle and the endochondral elements of the forelimb (Wright et al., 1995), suggesting that
non-coding sequence conserved with human and mouse have been identified flanking fugu Sox9a (Bagheri-Fam et al., 2001; Koopman et al., 2004), and these are candidates for regulatory elements driving expression in the limb and vertebral column (Wunderle et al., 1998). The subfunction partitioning model would predict that sox9a and sox9b in zebrafish would have different conserved sequences, and that comparative analysis would reveal candidates for these elements. Partitioned conserved non-coding sequences have already been identified for some zebrafish duplicates of mammalian genes (Postlethwait et al., 2004). Morpholino knockdown experiments for zebrafish duplicates of human HOXB1 have shown that the two fish duplicated genes have distinct and partially redundant functions, and study of conserved non-coding sequences has shown that the co-orthologs have suffered complementary degenerative mutations (McClintock et al., 2002).

The b971 mutation deletes sox9b

Our reverse genetics mutation screen identified a terminal chromosome deletion that removed sox9b and surrounding loci. We analyzed all known loci in this region (http://zfin.org/cgi-bin/mapper_select.cgi) for those that might play a role in the phenotype, and only sox8 (Cresko et al., 2003) is an obvious candidate for crest development (Cheung and Briscoe, 2003). Because sox8 is not expressed in neural crest derivatives in wild-type zebrafish embryos, and homozygous Sox8 knockout mice survive with no abnormalities in neural crest derivatives (Sock et al., 2001), we conclude that sox8 does not contribute to the phenotypes we observe.

To further test whether the sox9b^b971 phenotype is due to lack of sox9b, we increased wild-type sox9b activity in mutants and decreased sox9b activity in wild types. Splice-blocking sox9b MOs phenocopied each aspect of the sox9b^b971 phenotype, as expected if sox9b loss is responsible for the phenotypes we observed. Reciprocally, mRNA injections mosaically rescued Alcian blue-stained cartilage in sox9b^b971 mutants, despite the late phenotype, which gives ample opportunity for mRNA dilution and degradation. These tests confirmed that the effects we observed are due to the lack of sox9b rather than adjacent loci.

The role of sox9 genes in the genetic hierarchy of neural crest development

According to a recent model, neural folds originate at a threshold level of a BMP gradient and form neural crest due to Wnt, FGF, and retinoic acid signals (Aybar et al., 2002; Lewis et al., 2004; Tribulo et al., 2003). Early cranial crest expresses sox9b at high levels in the early neural crest in both the head and trunk as in tetrapod embryos, but they express sox9a at much lower levels than sox9b in that domain. In contrast, zebrafish embryos express sox9a at high levels in the somites as in tetrapods (Healy et al., 1999; Peters et al., 1999), but sox9b is not expressed in this domain. Together, these data show that the expression patterns of sox9a and sox9b tend to sum to the expression pattern in the mouse.

Parsimony suggests that expression domains shared by tetrapod and teleost Sox9 genes probably existed 450 million years ago in their last common ancestor. This supports the conclusion that specific independent regulatory elements were driving those expression domains in the last common ancestor of fish and tetrapods, and that those elements became partitioned between the two zebrafish genes over time (Force et al., 1999; Hughes, 1994; Postlethwait et al., 2004; Stoltzfus, 1999). Comparative sequence analysis can identify candidates for such regulatory elements. For example, five regions of evolution has divided up ancestral roles of Sox9 between zebrafish co-orthologs. Likewise, sox9a and sox9b are both expressed in the arches, but we show here that these are in different cell types, either the central mesenchyme and perichondrium, or the surrounding epithelial sheath. Embryos express sox9b at high levels in the early neural crest in both the head and trunk as in tetrapod embryos, but they express sox9a at much lower levels than sox9b in that domain. In contrast, zebrafish embryos express sox9a at high levels in the somites as in tetrapods (Healy et al., 1999; Peters et al., 1999), but sox9b is not expressed in this domain. Together, these data show that the expression patterns of sox9a and sox9b tend to sum to the expression pattern in the mouse.

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neural crest, or each gene is controlled by different factors in parallel pathways. After cranial crest begins to migrate, expression domains of sox9a and sox9b decrease in tgfpa2a mutants (Barrallo-Gimeno et al., 2004; Knight et al., 2004). This shows that tgfpa2a is required to establish the full range of sox9a- and sox9b-expressing cells.

In tetrapods, Snail is expressed in about the same temporal and spatial pattern as Sox9 and is the earliest known gene expressed in Xenopus neural crest (Essex et al., 1993; Spokony et al., 2002). Zebrafish has two co-orthologs of Snail, called snail1a and snail1b (formerly sna2), as well as snail2 (formerly slug) (Locascio et al., 2002), but only snail1b is expressed at high levels in premigratory neural crest (Locascio et al., 2002; Thiese et al., 1993; Thiese et al., 1995). Because the snail1b expression domain in premigratory crest was reduced in zebrafish embryos lacking sox9b function, we conclude that sox9b, but not sox9a mutants, showing that sox9b activity is not required for cranial crest migration, but that sox9b function is essential to establish the full size of the dlx2-expressing cell population.

Sox10 is expressed in most premigratory neural crest in a subpopulation of trunk crest cells that migrate in the medial pathway (Dutton et al., 2001) and in postmigratory craniofacial precursors (Kelsh and Raible, 2002). Likewise, crestin is expressed in premigratory and migratory neural crest, overlapping the initial sox10 and sox9b expression pattern (Li et al., 2002; Luo et al., 2001; Rubinstein et al., 2000). Activity of sox9b, but not sox9a, was necessary for full ventral migration of sox10- and crestin-expressing cells in the trunk and tail, but apparently not in the head.

These experiments, taken with results from tetrapods (Cheung and Briscoe, 2003; Mori-Akiyama et al., 2003; Spokony et al., 2002), show that Sox9 activity is essential for the establishment of the chondrogenic crest in vertebrates generally. On the other hand, among pigment cells, sox9 appears to act only in iridophore development. Although melanocytes appear enlarged in sox9b mutants, melanocyte precursors were normal. The melanocyte phenotype may be due to defective eyes in sox9 mutants because blind fish secrete more alpha-melanocyte stimulating hormone, which causes them to become more darkly pigmented than normal fish (Rodrigues and Sumpter, 1984).

The roles of zebrafish sox9 genes in skeletogenesis

In mammals, Sox9 protein binds to a chondrocyte-specific enhancer in an intron of Col2a1, and stimulates its transcription (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997; Zhou et al., 1998). Zebrafish col2a1 is co-expressed in several domains with sox9a and sox9b. Reduction of col2a1 expression in mutants correlated well with the sox9 co-ortholog predominantly expressed in each domain. In regions where the sox9 duplicates were co-expressed, double mutants summered the effect of the two homozygous mutations on col2a1 expression. This indicates that sox9a and sox9b act in the differentiation of chondrocytes in similar ways in overlapping sets of cartilaginous cells.

In the pectoral fin, sox9 co-orthologs have additive and exclusive roles. The scapulocoracoid, a chondral bone, expresses sox9a at high levels, and the sox9a mutant lacks this skeletal element. Likewise, human campomelic dysplasia patients are SOX9 heterozygotes and have hypoplastic scapulae (Houston et al., 1983; Mortier et al., 1997). In contrast, sox9b is not expressed in the scapulocoracoid, and this element is nearly normal in the sox9b mutant. The fin’s endochondral disc is nearly normal in sox9a mutants, but the distal portion is greatly reduced in sox9b mutants, again reflecting gene expression patterns. In the proximal portion of the endochondral disc, the two sox9 orthologs play additive roles because the double mutant lacks this cartilage. Double mutants retain Alcian staining in the anlage of the cleithrum, a dermal bone.

Zebrafish sox9 co-orthologs play different roles in chondrocyte morphogenesis and survival. By imaging live developing embryos, we confirmed that sox9a does not regulate prechondrocyte cell number or condensation (a role of Sox9 in mouse (Bi et al., 2001)), but does control the stacking of chondrocytes in orderly cell rows (Yan et al., 2002). On the other hand, sox9b does not control stacking, but is necessary for pharyngeal cartilages to acquire their normal cell number, either by suppressing cell death or increasing cell proliferation. Although chondrocytes stack in sox9b mutants, individual pharyngeal cartilage organs are not well shaped, showing that sox9b activity is important for fine aspects of cartilage patterning.

Analysis of zebrafish mutants showed that sox9a and sox9b play different roles in development of the bony skeleton. The sox9a gene is expressed in the mesenchyme and perichondrium of branchial arches, while sox9b is expressed in the epithelium that surrounds each arch, and is required for the differentiation of these cartilages into bone. This suggests that a sox9b-dependent signal emanating from the pharyngeal pouch epithelium may trigger the sox9a-expressing perichondrium to differentiate into osteoblasts and form bone.

Runx2 encodes a transcription factor expressed in prehypertrophic and hypertrophic chondrocytes and osteoblasts (Takeda et al., 2001), and it promotes chondrocyte maturation and osteoblast differentiation (Inada et al., 1999; Komori et al., 1997; Otto et al., 1997). Expression of runx2b was normal in sox9a mutants, but was severely reduced in the arches of sox9b mutants, suggesting that sox9b exerts its effect on bone formation by supporting runx2b expression. This suggests that sox9b in the arch epithelium, but not sox9a in the central chondrocytes, is required for runx2b expression in the perichondrium, which in turn is necessary for the perichondrium to differentiate into osteocytes and finally bone. Only a portion of two dermal bones, the opercle in the craniofacial skeleton and the cleithrum in the pectoral girdle, are largely independent of sox9 activity.

Chimeric mice bearing Sox9-deficient cells also show interactions of Runx2 and Sox9. Depleting Sox9 in the nasal region causes an expansion of the Runx2 expression domain, suggesting that Sox9 negatively regulates Runx2 in these cells.
In contrast, Sox9-deficient mouse limb buds lack Runx2 expression, suggesting that Sox9 positively regulates Runx2 in limb buds. Sox9 may positively regulate Runx2 in cases in which Sox9-deficient cells fail to complete chondrocyte differentiation and become osteoblasts (Mori-Akiyama et al., 2003). In other situations, lack of Sox9 activity may block cells from later steps necessary for bone formation. Reciprocally, Sox9 expression is expanded in mouse tissue culture cells lacking Runx2 and in the limbs of Runx2 mouse mutants, showing that Runx2 is a negative regulator of Sox9 (Stock et al., 2004; Yoshida et al., 2002). While Runx2 is expressed in the perichondrium in mouse (Bronckers et al., 2003), Sox9 is not (Smits et al., 2001), demonstrating that in this tissue, Sox9 cannot be a direct transcription factor for Runx2. Subfunction partitioning between the two recently described Runx2 co-orthologs in zebrafish (Flores et al., 2004) may facilitate analysis of these problems.

Null mutations in genes that are essential at several developmental stages (pleiotropy) will block development at the earliest essential subfunction, which can obscure later gene roles. In some cases, conserved temporal subfunctions have partitioned between teleost duplicates. For example mouse Nodal mutants are blocked in gastrulation (Conlon et al., 1994; Varlet et al., 1997), masking later gene functions. Zebrafish has two Nodal genes (Blader and Strähle, 1998; Dougan et al., 2003; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998), and mutant analysis reveals both early and late functions. Likewise, in Xenopus Sox9 knockdown embryos, a role for Sox9 in chondrocyte stacking was obscured because early Sox9 function (which in zebrafish partitions to sox9b) is required for the formation of the cells that stack (Spokony et al., 2002). Thus, subfunction partitioning in zebrafish permitted ready identification of late gene functions obscured in tetrapods by early gene functions and by haploinsufficiency.

Subfunction partitioning, sox9 and sox10

sox8, sox9 and sox10 belong to the Sox family gene family and are expressed in overlapping domains in neural crest, heart, limb buds, glia, and gonads (Bell et al., 2000; Bowles et al., 2000; Cheng et al., 2001; Chimal-Monroy et al., 2003; Schepers et al., 2000; Sock et al., 2001). Premigratory crest expresses both Sox9 and Sox10, but Sox9 is required for ectomesenchymal crest, and Sox10 for non-ectomesenchymal crest (Akiyama et al., 2002; Dutton et al., 2001). We propose that an ancestral non-vertebrate chordate possessed a single Sox9/10 gene that was expressed in cells at the neural plate border and was essential for specifying pigment cells, mesenchyme, glia, and some neurons (see Jeffery et al., 2004). After gene duplication prior to the divergence of zebrafish and human lineages (Bowles et al., 2000; Chiang et al., 2001; Dutton et al., 2001; Li et al., 2002), subfunction partitioning (Force et al., 1999) may have resulted in ectomesenchymal functions largely partitioning to Sox9, and non-ectomesenchymal functions mainly segregating to Sox10, but with some overlap due to the retention of a few ancestral subfunctions by both genes. This proposed process is analogous to subfunction partitioning between the zebrafish sox9 duplicates documented here. Because the advent of neural crest is a key to the evolution of vertebrates, the duplication of SoxE and subfunction partitioning among its descendant genes may have been a crucial step in vertebrate origins. This hypothesis makes specific predictions about the roles of SoxE in the development of non-vertebrate chordates that have yet to be tested.

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