Specification of the otic placode depends on Sox9 function in *Xenopus*

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Summary

The vertebrate inner ear develops from a thickening of the embryonic ectoderm, adjacent to the hindbrain, known as the otic placode. All components of the inner ear derive from the embryonic otic placode. Sox proteins form a large class of transcriptional regulators implicated in the control of a variety of developmental processes. One member of this family, Sox9, is expressed in the developing inner ear, but little is known about the early function of Sox9 in this tissue. We report the functional analysis of Sox9 during development of *Xenopus* inner ear. Sox9 otic expression is initiated shortly after gastrulation in the sensory layer of the ectoderm, in a bilateral patch of cells immediately adjacent to the cranial neural crest. In the otic placode, Sox9 colocalizes with Pax8 one of the earliest gene expressed in response to otic placode inducing signals.

Depletion of Sox9 protein in whole embryos using morpholino antisense oligonucleotides causes a dramatic loss of the early otic placode markers Pax8 and Tbx2. Later in embryogenesis, Sox9 morpholino-injected embryos lack a morphologically recognizable otic vesicle and fail to express late otic markers (Tbx2, Bmp4, Otx2 and Wnt3a) that normally exhibit regionalized expression pattern throughout the otocyst. Using a hormone inducible inhibitory mutant of Sox9, we demonstrate that Sox9 function is required for otic placode specification but not for its subsequent patterning. We propose that Sox9 is one of the key regulators of inner ear specification in *Xenopus*.

Key words: Sox9, Tbx2, Pax8, Dlx3, Otic placode, Inner ear, Wnt, Fgf, *Xenopus*

Introduction

The vertebrate inner ear is a sensory organ implicated in hearing, balance and detection of acceleration. It develops from a thickening of the embryonic ectoderm, adjacent to the hindbrain, known as the otic placode. The placode invaginates to form first the otic cup and then the otic vesicle or otocyst. During this process, neuroblasts delaminate from the otic cup to form the vestibulo-acoustic ganglia. The otocyst will then undergo intense proliferation followed by a differentiation phase during which the development of the specific cell types of the inner ear will take place. With the exception of the pigment cells of the stria vascularis and the secretory epithelium of the cochlea, which are of neural crest origin, all components of the inner ear derive from the otic placode (Torres and Giraldez, 1998).

Classical transplantation experiments have shown that ear formation is controlled by interactions with adjacent tissues including the hindbrain and the paraxial mesoderm (Torres and Giraldez, 1998; Baker and Bronner-Fraser, 2001). Recent studies have implicated secreted factors of the Fgf family (Vendrell et al., 2000; Ladher et al., 2000; Phillips et al., 2001; Adamska et al., 2001; Leger and Brand, 2002; Liu et al., 2003; Wright and Mansour, 2003; Alvarez et al., 2003), Wnt8C (Ladhler et al., 2000), retinoic acid (Pasqualetti et al., 2001) and Shh (Riccomagno et al., 2002; Liu et al., 2002) in the

specification and patterning of the inner ear in the vertebrate embryo.

The otocyst can be divided along its dorsoventral axis into three functional domains. Ventrally, the cochlear region is responsible for the sense of hearing; the medial region is implicated in the senses of motion and position; and the dorsal region is involved in vestibular functions. In the recent years, a number of transcription factors that show restricted expression pattern in each of these functional domains have been identified. These genes include *Pax8*, *Pax2*, *Otx1*, *Prx1*, *Prx2*, *Six1*, *Tbx2* and *Hmx3* (reviewed by Torres and Giraldez, 1998; Baker and Bronner-Fraser, 2001). In the mouse, mutations in some of these genes resulted in the loss of specific inner ear components, demonstrating the importance of some of these transcription factors in the specification and patterning of the inner ear (reviewed by Fekete, 1999).

The Sox proteins constitute a large family of transcriptional regulators (Wegner, 1999). They have been implicated in the control of a broad range of developmental processes (Kamachi et al., 2000). One member of this family, Sox9, has been shown to regulate chondrogenesis in the mouse embryo (Wright et al., 1995; Bell et al., 1997; Bi et al., 1999; Bi et al., 2001). Mutations in one Sox9 allele result in campomelic dysplasia, a lethal human disorder characterized by autosomal XY sex reversal and severe skeletal malformations (Houston et al.,

1983; Foster et al., 1994; Wagner et al., 1994). These symptoms have also been associated with deafness (Houston et al., 1983; Savarirayan et al., 2003), consistent with Sox9 expression in the otic epithelium of several species (Zhao et al., 1997; Ng et al., 1997; Spokony et al., 2002; Li et al., 2002; Liu et al., 2003). However, very little is known about the function of Sox9 during development of the auditory system. We report experiments in *Xenopus* embryos that analyze the function of Sox9 during inner ear development and that establishes Sox9 as a key regulator of otic placode specification.

Materials and methods

Constructs

An inhibitory mutant of Sox9 (Sox9ΔC), in which the C-terminal domain of XSox9 cDNA was deleted at amino acid 311, was generated by PCR (forward primer, ATCGAT GCCACC-ATGAATCTCTTGGATCCC; reverse primer, CTCGAGCTGAGT-GGAGCC CAACCCCTG) and ligated into pGEMT. A hormone-inducible construct was generated by fusing Sox9ΔC to the coding region of the human glucocorticoid receptor ligand-binding domain (GR) as described (Gammill and Sive, 1997; Tada et al., 1997). This fusion construct cloned into pCS2+ is referred as Sox9ΔC-GR. Mouse Sox9 pCMV-SPORT6 (accession number BC023796) was obtained from Open Biosystems and the ORF subcloned by PCR into the *Cla*I and *Xho*I sites of pCS2+. All expression constructs were sequenced and the corresponding protein monitored using an in vitro transcription/translation-coupled rabbit reticulocyte lysate system in the presence of [^{35}S] methionine (Promega) and resolved on a NuPAGE BIS-Tris gel (Invitrogen).

Embryo injections and dexamethasone treatment

Dominant-negative Fgf receptor XFD/FgfR (Amaya et al., 1991) (2 ng), GSK3β (Saint-Jeannet et al., 1997) (1 ng), Sox9ΔC-GR (1 ng) and mouse Sox9 (2 ng) mRNAs were synthesized in vitro using the Message Machine kit (Ambion, Austin, TX). Sox9 morpholino antisense (Sox9-mo, 5-10 ng, GCAAAAATGGGGAAAGGTAA-GAAAG) (Spokony et al., 2002), 5 bp mismatched Sox9 morpholino antisense (Sox9-mis, 10 ng, G<u>G</u>AAAAAT<u>C</u>GG<u>C</u>AAAG<u>C</u>TAA<u>C</u>A-AAG), standard control morpholino antisense (Co-mo, 10 ng), and Dlx3 morpholino antisense (Dlx3-mo, 30 ng, ATAGTTTATTA-CCTGCGTCTGAGTG) were purchased from GeneTools (Corvallis, OR). Synthetic mRNAs and morpholinos were injected in one blastomere at the two-cell or eight-cell stage as described (Saint-Jeannet et al., 1994; Saint-Jeannet et al., 1997; Spokony et al., 2002). At the eight-cell stage, one animal ventral cell was injected to target the otic placode territory. Embryos injected with Sox9ΔC-GR mRNA were treated at different time points (stage 6, 11, 15 or 20) with 10 μM of dexamethasone (Sigma) in NAM 0.1$\times$ as described (Gammill and Sive, 1997; Tada et al., 1997; Aoki et al., 2003).

Lineage tracing and whole-mount in situ hybridization

In all experiments, embryos were co-injected with β-galactosidase mRNA (β-gal, 1 ng). At stage 22 or 35 embryos were fixed in MEMFA (Harland, 1991) and successively processed for Red-Gal (Research Organics) staining and in situ hybridization. For the Dlx3-mo injections, a fluorescein-labeled control morpholino (Genetools) was co-injected and used to identify the injected side in a fluorescence microscope. Antisense DIG-labeled probes (Genius kit, Roche) were synthesized using template cDNA encoding Tbx2 (Hayata et al., 1999; Takabatake et al., 2000), Pax8 (Heller and Brandli, 1999), Otx2 (Pannese et al., 1995), Bmp4 (Jones et al., 1992; Kil and Collazo, 2001), Xwnt3a (Wolda et al., 1993) and Sox9 (Spokony et al., 2002). Whole-mount in situ hybridization was performed as described (Harland, 1991). For histology, stained embryos were embedded into Paraplast+, 12 μm sections cut on a rotary microtome and counterstained with Eosin.

Western blot analysis

Sox9ΔC-GR-injected embryos were collected at different stages, homogenized, resolved on a NuPAGE BIS-Tris gel and blotted onto nitrocellulose. Blots were subsequently incubated in the presence of a Sox9 polyclonal antibody (P-20, Santa Cruz Biotechnology) at a 1:500 dilution, washed and incubated with anti-goat Ig coupled to horseradish peroxidase (Santa Cruz Biotechnology, 1:60,000 dilution). The product of the reaction was revealed using the SuperSignal West Femto Maximum Sensitivity Substrate from Pierce and detected by exposure onto a BioMax film (Kodak). Blots were stripped according to the manufacturer recommendations (Pierce) and probed with anti-α-tubulin antibody (T-9026, Sigma; 1:500 dilution) as a loading control.

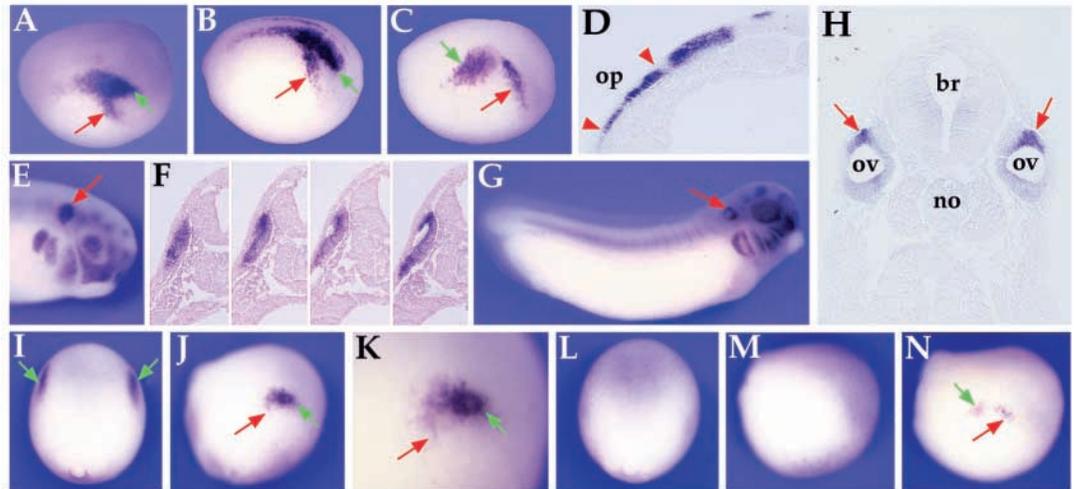## Results

Expression of Sox9 in the developing inner ear

We analyzed Sox9 expression pattern in the developing otic placode and vesicle by whole-mount in situ hybridization. At the late early neurula stage staining accumulates at the lateral edges of the neural plate (green arrow, Fig. 1A), in the prospective tissue of the neural crest as previously described (Spokony et al., 2002). Sox9 is also detected in a bilateral patch of cells (red arrow, Fig. 1A) immediately adjacent to the lateral neural crest and corresponding to the prospective otic placode. Sox9 expression in the inner ear forming region at stage 14 (red arrow, Fig. 1B) colocalizes with that of Pax8 (Heller and Brandli, 1999) (Fig. 1C). Tissue sections of stage 14 embryos highlight the neural crest expression of Sox9 as well as Sox9 expression in the prospective otic placode that appears to be confined to the sensory layer of the ectoderm (Fig. 1D). By the tailbud stage, the diffuse expression of Sox9 in the presumptive otic placode has become more coherent and restricted to the otic cup (Fig. 1E,F). As the otic cup invaginates to form a vesicle, Sox9 is expressed throughout the otic vesicle with more intense staining at the dorsal most region of the otic vesicle, prospective region of the semicircular canals and endolymphatic duct (Fig. 1G,H).

To determine more precisely whether Sox9 expression precedes that of Pax8 in the presumptive otic territory, in situ hybridization for both genes was performed on stage-matched embryos. Although Sox9 is first detected at stage 12.5 in the prospective otic tissue ventral to the neural crest expression domain (Fig. 1I-K), Pax8 is detected only at stage 13 in both the prospective otic placode and pronephros (Fig. 1L-N). Sox9 is therefore one of the earliest genes expressed in the presumptive otic placode, consistent with a potential function in inner ear development.

Sox9 expression in the otic placode is regulated by Wnt and Fgf signaling

Wnt and fibroblast growth factor (Fgf) signaling pathways have been implicated in inner ear formation in the mouse, chick and zebrafish embryos (Ladher et al., 2000; Leger and Brand, 2002; Liu et al., 2003; Wright and Mansour, 2003); therefore, we tested the requirement of these signaling pathways for Sox9 expression in the otic placode/vesicle in *Xenopus*. Injection of glycogen synthase kinase 3β (GSK3β) mRNA, a downstream component of the canonical Wnt

Fig. 1. Sox9 whole-mount in situ hybridization. (A) Sox9 expression at early neurula stage (stage 3), dorsolateral view, anterior towards the right. Sox9 is detected in two major domains, the neural crest-forming regions (green arrows) and the prospective otic placode (red arrows). (B) Sox9 expression is greatly increased in the neural crest (green arrows) and in the prospective otic placode (red arrows) at stage 14. Lateral view, anterior towards right. (C) For comparison, Pax8 expression at the neurula stage is shown. Pax8 is detected in the prospective pronephros (green arrow) and the otic placode (red arrow). Lateral view, anterior towards the right. (D) Cross-section through the prospective otic placode of a stage 14 embryo, Sox9 is detected in the sensory layer of the ectoderm (op, red arrowheads) before any sign of thickening of the ectoderm. (E) At the tailbud stage, Sox9 persists in the invaginating otic placode (red arrow). Lateral view, anterior towards the right. (F) Serial cross-sections of a tailbud stage embryo; Sox9 is expressed throughout the invaginating otic cup. (G) Stage 30 embryo, lateral view, anterior towards the right; (H) corresponding section. Sox9 expression is detected in the dorsal region of the otic vesicle (ov, red arrows). (I-M) In stage-matched embryos, Sox9 is first detected in the presumptive otic ectoderm (red arrows) lateral to the prospective neural crest (green arrows) at stage 12.5 (J,K). (K) Higher magnification view of the embryo in J. Sox9 expression appears prior to that of Pax8 (L,M), which is initiated in the presumptive otic ectoderm (red arrow) and pronephros (green arrow) at stage 13 (N). Dorsal views, anterior towards the top (I,L). Lateral views, anterior towards the right (J,M,N).

pathway known to antagonize Wnt signaling (He et al., 1995), completely blocked Sox9 expression in the otic placode at stage 17 (Fig. 2A, upper panel), in 84% of injected embryos (*n*=68). LRP6 is a component of the Wnt receptor complex. Similar results (not shown) were obtained by injection of a dominant-negative form of LRP6 (LRP6ΔC) known to block Wnt signaling in *Xenopus* (Tamai et al., 2000). Although the neural crest expression domain of Sox9 was also altered in these embryos injected with GSK3β (Luo et al., 2003) or LRP6ΔC (not shown), the otic placode component of Sox9 expression was always the first domain to be affected. Expression of a dominant-negative Fgf receptor (XFD/FgfR), lacking the cytoplasmic domain, has been shown to block Fgf signaling in vivo (Amaya et al., 1991). Approximately 45% of XFD/FgfR-injected embryos (*n*=60) exhibited a reduction of Sox9 expression in the otic vesicle at stage 23 (Fig. 2A, lower panel). As previously described, these embryos had also an open blastopore dorsally, reflecting Fgf requirement for the development of posterior structures (Amaya et al., 1991).

To further investigate the consequence of inhibiting Wnt and Fgf signaling on the development of the otic placode, histological sections of GSK3- and XFD-injected embryos at stage 35 were performed. Interestingly, in both cases these embryos appear to form an otic vesicle, however considerably reduced in size (Fig. 2B). This result may indicate that only an incomplete inhibition of Fgf and Wnt signaling pathways was achieved in GSK3- and XFD-injected embryos, or that other factors are also required for the induction of the otic placode.

These experiments indicate that the otic expression of Sox9 is under the positive control of both Wnt and Fgf, and suggest that these signaling pathways are implicated in the development of the otic vesicle in *Xenopus*.
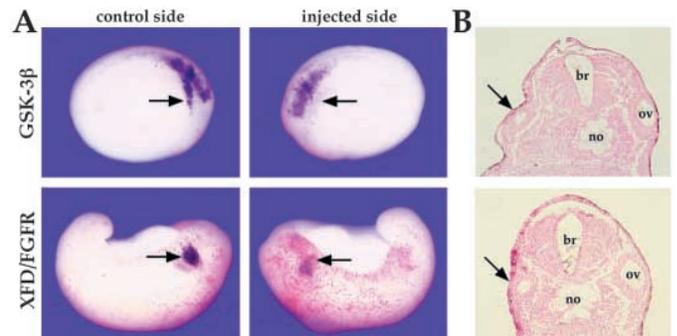
Fig. 2. Inhibition of Wnt or Fgf signaling pathway blocks Sox9 expression in the otic placode/vesicle. (A) Sox9 expression is lost in the otic placode (upper panels, stage 17) of embryos injected with 1ng of GSK3β mRNA (arrows, injected side). Sox9 expression is reduced in the otic vesicle (lower panels, stage 23) of embryos injected with 1 ng of dominant-negative FgfR (XFD) mRNA (arrows, injected side). For comparison, normal expression of Sox9 is shown on the uninjected side of these embryos (arrows, uninjected side) at stage 17 and stage 23. As Fgf signaling is also required for development of posterior structures, XFD/FgfR-injected present a characteristic open blastopore (lower panels). All panels are lateral views, anterior towards the right (left panels) or anterior towards the left (right panels). (B) Transverse section through an embryo injected with GSK3β (upper panel) or XFD/FgfR (lower panel) mRNA. In both cases the embryos present a reduced otic vesicle on the injected side (left side, arrows). br, brain; no, notochord; ov, otic vesicle.

Sox9 depletion prevents otic placode and vesicle formation

To address Sox9 function during inner ear development we performed loss-of-function studies using morpholino

Fig. 3. Sox9 depletion prevents formation of the otic placode. (A) At stage 22, Pax8 and Tbx2 expression is eliminated upon injection of 10 ng of Sox9-mo in one blastomere at the two-cell stage (left side). For comparison, the otic expression of Pax8 and Tbx2 is unaffected (arrows) in embryos injected with 10 ng of Co-mo or Sox9-mis oligos. Sox9-mo phenotype can be rescued by injection of 2 ng of mouse Sox9 mRNA (Sox9-mo+mSox9). RNA encoding the lineage tracer β-galactosidase was co-injected with the morpholino oligo to identify the injected side (red staining, left side in all panels). (B) Quantification of Tbx2 and Pax8 in situ hybridization results. The numbers at the top of each bar indicate the number of cases analyzed.
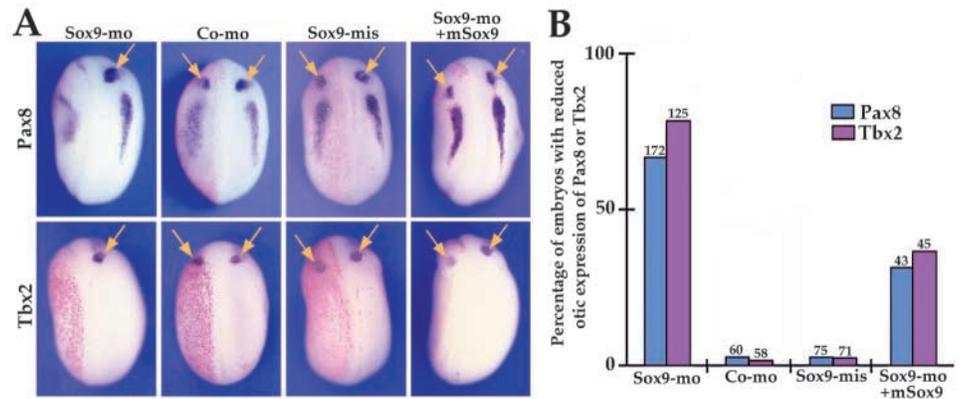

Fig. 4. Sox9 depletion prevents expression of late otic vesicle markers. (A) Schematic representation of the expression pattern (purple color) of four markers genes expressed in the epithelium of the otic vesicle at stage 35. Tbx2 is expressed through out the entire otic vesicle. Bmp4 expression is restricted to the anterior and posterior region of the otic vesicle. Wnt3a and Otx2 are expressed at the dorsal and ventral aspect of the otic vesicle, respectively. d, dorsal; v, ventral; a, anterior; p, posterior. (B) Tbx2, Bmp4, Wnt3a and Otx2 expression is lost in the otic vesicle of embryos injected with 2 ng of Sox9-mo in one blastomere at the eight-cell stage (arrows, injected side). RNA encoding the lineage tracer β-galactosidase was co-injected with the oligo AS to identify the injected side (red staining). For comparison, normal expression of Tbx2, Bmp4, Wnt3a and Otx2 is shown on the uninjected side of these embryos (arrows, control side). (C,D) Transverse section through an embryo injected with 2 ng of Sox9-mo. On the injected side (left side, arrow), the otic vesicle fails to form. br, brain; no, notochord; ov, otic vesicle.
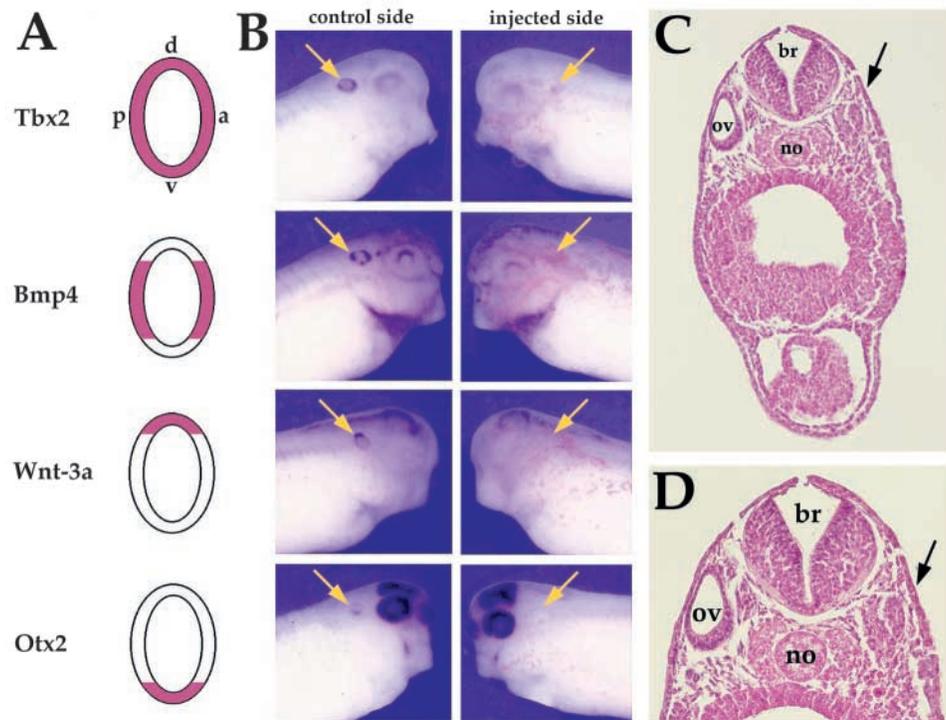
antisense oligonucleotides (Sox9-mo). The characteristics and the specificity of this antisense oligo have been previously described (Spokony et al., 2002). Embryos at the two-cell stage were injected in one blastomere with 10 ng of Sox9-mo, a 5 bp mismatched antisense (Sox9-mis) or a standard control oligo (Co-mo) together with RNA encoding the lineage tracer β-galactosidase. In situ hybridization analysis revealed that a large proportion of Sox9-mo-injected embryos failed to express early otic placode markers such as Pax8 (66% reduced, n=172) and Tbx2 (77% reduced, n=125) as shown in Fig. 3. By contrast, the expression of both genes was unperturbed in Sox9-mis- or Co-mo-injected embryos (Fig. 3A,B). Importantly, the otic expression of Pax8 and Tbx2 can be rescued in Sox9-depleted embryos by co-injection of wild-type mouse Sox9; injection of mouse Sox9 mRNA decreased the number of embryos with

reduced Pax8 or Tbx2 expression by approximately 50% (Fig. 3A,B).

To further characterize the phenotype of Sox9-depleted embryos, we have analyzed the expression of four late markers genes Tbx2, Bmp4, Wnt3a and Otx2 in Sox9-mo injected embryos. These genes exhibit restricted expression pattern along the dorsoventral and anteroposterior axes of the otic vesicle (Fig. 4A). Embryos were injected with 2 ng of Sox9-mo or Co-mo in one animal ventral blastomere at the eight-cell stage and analyzed by in situ hybridization at stage 35. Upon injection of Sox9-mo, all four markers were greatly reduced (Fig. 4B) in ~75% of the injected embryos (Tbx2, n=51; Bmp4, n=81; Wnt3a, n=68; Otx2, n=77). Injections of control morpholinos had no effects on the otic vesicle expression of these markers (not shown). Moreover, sagittal section through Sox9-mo-injected embryos revealed that most of these

Fig. 5. Hormone inducible dominant-negative Sox9 construct. (A) Schematic representation of Sox9, Sox9ΔC and Sox9ΔC-GR fusion constructs. Sox9 lacking the transactivation domain (Sox9ΔC) is fused to the ligand-binding domain of human glucocorticoid receptor (hGR). The HMG box is highlighted in blue, and the putative transactivation domain is underlined in red. (B) Western blot analysis of Sox9ΔC-GR protein in extracts from Sox9ΔC-GR-injected embryos collected at different stages. The fusion protein is expressed at similar levels at all stages (stage 10-20). This Sox9 antibody directed against the N-terminal domain of Sox9 does not allow detection of endogenous Sox9 protein (control, uninjected embryo). Tubulin is presented as a loading control.
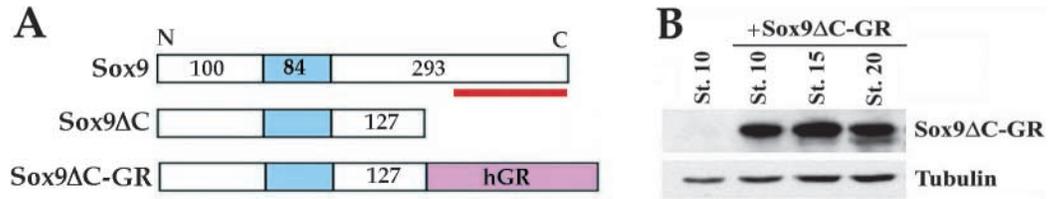
embryos lacked a recognizable otic vesicle on the injected side, while the overall morphology of these embryos is otherwise unperturbed (Fig. 4C,D).

Altogether, these results indicate that Sox9 is an essential regulator of inner ear formation, and further support the existence of a Sox9-dependent pathway required for otic placode development.

Sox9 is required to specify the otic placode but not for its subsequent patterning

We next decided to determine the window of time during which Sox9 is required for otic placode development. To this end, we generated an inducible inhibitory mutant Sox9 construct (Sox9ΔC-GR) in which the hormone-binding domain of the human glucocorticoid receptor is fused to a form of Sox9 lacking the transactivation domain (Fig. 5A). This construct was generated based on the observation that numerous Sox9 mutations in human affected by campomelic dysplasia are due to truncations or frameshifts that eliminate the transactivation domain at the C terminus, resulting in a loss of transactivation activity, such truncated proteins are believed to interfere with wild-type Sox9 function (McDowall et al., 1999; Preiss et al., 2001). This type of inducible construct allows temporal inactivation of Sox9 function by addition of dexamethasone at specific times during embryogenesis (Gammill and Sive, 1997; Tada et al., 1997). By western blot analysis, using a Sox9-specific antibody, we showed that after injection at the two-cell stage the levels of Sox9ΔC-GR protein remained constant throughout development, from stage 10 to 20 (Fig. 5B).

Embryos were injected in one blastomere at the two-cell stage with 1 ng of Sox9ΔC-GR mRNA and treated with dexamethasone at various stages (Fig. 6A). Addition of dexamethasone at the blastula stage (stage 6) caused a loss of otic expression of Pax8 and Tbx2 at stage 22 (Fig. 6B,C). This result is consistent with the phenotype of Sox9-depleted embryos described earlier (Fig. 3) and suggests that this fusion construct is fully active in blocking Sox9 function. Dexamethasone treatment at the gastrula stage (stage 11) also resulted in a similar loss of both otic placode markers (Fig. 6B,C). However, inactivation of Sox9 at neurula stage (+Dex stage 15) failed to block Pax8 and Tbx2 expression in the developing otic placode (Fig. 6B,C). This result indicates that Sox9 function is required prior to stage 15 to specify the otic placode.

As Sox9 is also expressed the developing eyes and in the genital ridges (Spokony et al., 2002), defects in Tbx2 and Pax8
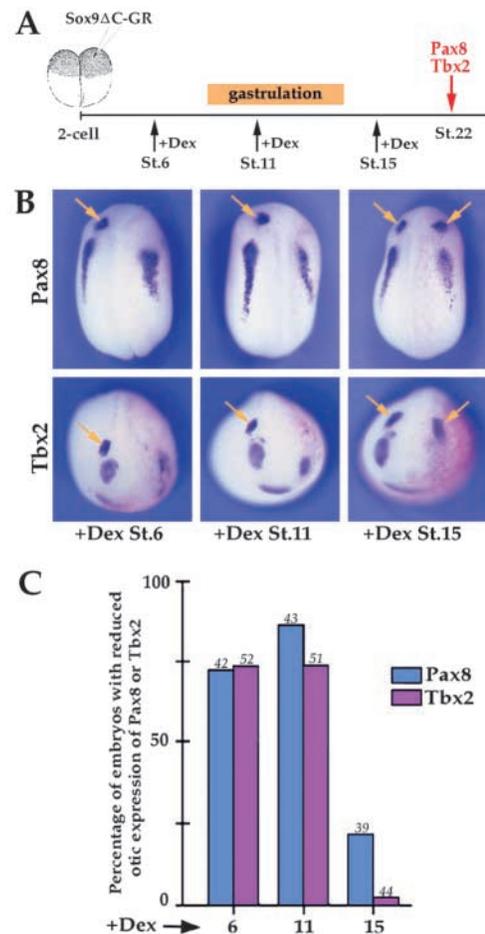

Fig. 6. Sox9 is required to specify the otic placode. (A) Experimental procedure. Embryos at the two-cell stage are injected in one blastomere with 1 ng of Sox9ΔC-GR mRNA. Embryos are subsequently incubated with dexamethasone (+Dex) at different time points during development (stage 6, 11 and 15), and fixed at stage 22 for detection of Pax8 and Tbx2 by whole-mount in situ hybridization. (B) Addition of dexamethasone at the blastula (stage 6) and gastrula (stage 11) stages results in a severe reduction of Pax8 and Tbx2 expression domain in the otic placode. However, Pax8 and Tbx2 otic expression remain unaffected in embryos treated with dexamethasone at stage 15 (arrows). Upper panels are dorsal views, anterior towards the top. Lower panels are anterior views. RNA encoding the lineage tracer β-galactosidase was co-injected to identify the injected side (red staining). In all panels, embryos are injected on the left side. (C) Quantification of the in situ hybridization results. The number of cases analyzed for each time point is indicated.
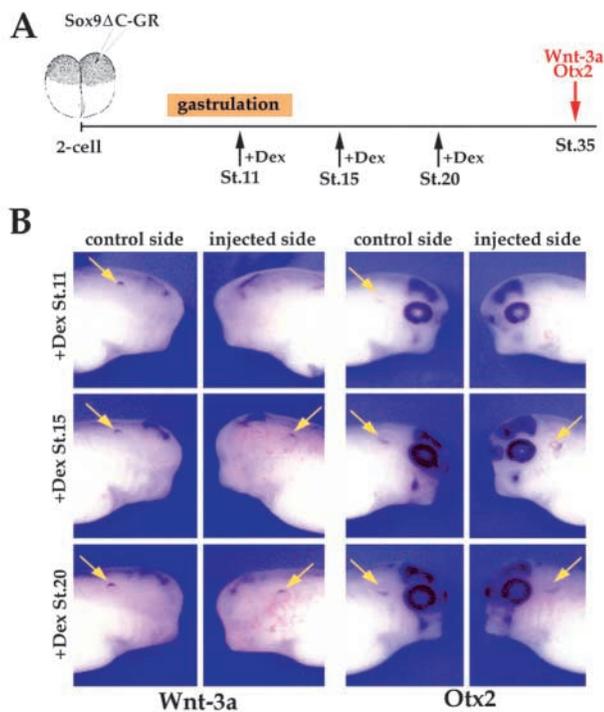
Fig. 7. Sox9 is not required to pattern the otic vesicle.
(A) Experimental procedure. Embryos at the two-cell stage are injected in one blastomere with 1 ng of Sox9ΔC-GR mRNA. Embryos are subsequently incubated with dexamethasone (+Dex) at different time points during development (stage 11, 15 and 20), and fixed at stage 35 for detection of Wnt3a and Otx2 by whole-mount in situ hybridization. (B) While addition of dexamethasone at stage 11 blocks Wnt3a and Otx2 expression on the injected side, addition of dexamethasone at the stage 15 or stage 20 does not affect the regionalized expression of Wnt3a and Otx2 (arrows). RNA encoding the lineage tracer β-galactosidase was co-injected to identify the injected side (red staining), the right side for all embryos.
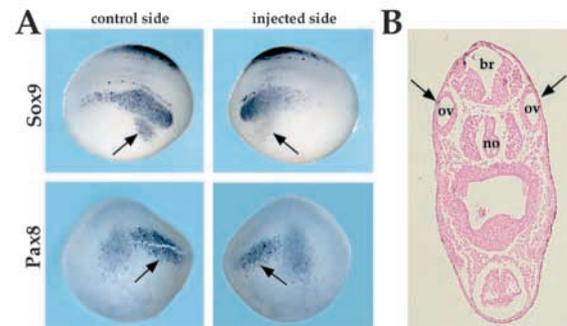

Fig. 8. Dlx3 depletion blocks the otic expression of Sox9.
(A) Injection of 30 ng of Dlx3-mo in one animal ventral blastomere at the two-cell stage reduces Sox9 otic expression (injected side, arrow). For comparison, the control side shows the normal expression of Sox9 in the presumptive otic placode (arrow). Pax8 expression was not significantly inhibited by this procedure. Lateral views of stage 14 embryos, anterior towards the right (left panel). (B) Dlx3-mo-injected embryos form normal otic vesicle at stage 35 (arrows). br, brain; no, notochord; ov, otic vesicle.

expression in the eye rudiment and in the prospective kidney, respectively, were also observed (Fig. 6B). Interestingly, in these tissues the timing of Sox9 requirement differs from that of the otic placode as Tbx2 and Pax8 expression was perturbed even when Sox9 was inactivated by addition of dexamethasone at the neurula stage (stage 15).

In order to determine whether Sox9 is also required for subsequent patterning of the otic placode/vesicle, we analyzed the otic expression of Otx2 and Wnt3a in stage 35 embryos in which Sox9 was inactivated at stage 15 or stage 20, thereby bypassing the early requirement for Sox9 (Fig. 7A). As expected embryos treated with dexamethasone at stage 11 lacked an otic vesicle on the injected side (Fig. 7B). However, most of the embryos treated at stage 15 or 20 developed normal otic vesicles with regionalized Otx2 (86%, n=43) and Wnt3a (85%, n=41) expression (Fig. 7B).

Both set of experiments indicate that Sox9 is strictly required for specification of the otic placode and that subsequent otic vesicle differentiation and patterning can occur independently of Sox9 function.

Maintenance of Sox9 expression depends on Dlx3

The distal-less related homeobox gene Dlx3 is expressed during the initial stages of sensory placode development and has been implicated in otic placode formation in the zebrafish embryo (Ekker et al., 1992; Solomon and Fritz, 2002; Liu et al., 2003). Therefore, we analyzed its requirement for Sox9, Pax8 and Tbx2 otic expression in *Xenopus* using a morpholino antisense oligonucleotide (Dlx3-mo) that inhibits splicing of the second intron from the Dlx3 transcript. Embryos that received unilateral injection of 30 ng of Dlx3-mo exhibited a specific reduction of Sox9 otic expression at the neurula stage (82% n=22), while Sox9 neural crest expression domain was only marginally affected in these embryos (Fig. 8). A similar result was obtained with a different Dlx3 morpholino targeted to the translational initiation site (80%, n=25; data not shown). Interestingly, Pax8 or Tbx2 otic expression (Fig. 8A and not shown) was not significantly disturbed by inhibiting Dlx3 expression (slight reduction of Pax8 in 31% of the embryos, n=39; no reduction of Tbx2, n=15); moreover, these embryos formed normal otic vesicles at stage 35 (Fig. 8B).

Altogether, these results are consistent with the view that Sox9 and Dlx3 function in the same regulatory pathway during otic placode formation, and that Dlx3 is required for maintenance of Sox9 expression following the initial function of Sox9 in otic placode specification.

Discussion

In this study, we present evidence that the transcriptional activator Sox9 is required for the specification of the otic placode in *Xenopus* embryos. This is supported by several observations: (1) Sox9 is expressed in the presumptive otic placode before it becomes morphologically distinct; (2) depletion of Sox9 by antisense oligonucleotides results in a loss of early and late otic markers and a failure to form a recognizable otic vesicle; and (3) temporal inactivation of Sox9 using an hormone inducible inhibitory mutant demonstrates that Sox9 is required for specification of the otic placode but not for its subsequent patterning.

Sox9 is one of the earliest genes expressed in the presumptive otic placode (stage 12/13), its expression is

initiated prior to any morphological changes in the sensory layer of the ectoderm. The dorsolateral thickening of the ectoderm that defines the position of the prospective otic placode becomes only visible a few hours later around stage 21/22 (Nieuwkoop and Farber, 1956). Spatially Sox9 and Pax8 have similar expression domain within the presumptive otic placode (Heller and Brandli, 1999), while temporally Sox9 otic expression appears to precede that of Pax8. This observation, together with the finding that Sox9-depleted embryos fail to express Pax8 suggests that Sox9 may function upstream of Pax8 during inner ear development. However, this does not exclude the possibility that Sox9 and Pax8 may also be involved in maintaining each other's expression in the presumptive otic placode.

In *Xenopus*, the distal-less related gene Dlx3 (Xdll2) is expressed in the ventral ectoderm at the gastrula stage and later marks the boundary between the ectoderm and the neural crest (Papalopulu and Kintner, 1993; Dirksen et al., 1994; Luo et al., 2001) (reviewed by Beanan and Sargent, 2000). This tissue encompasses the placodal ectoderm that will give rise to the olfactory and otic placodes. Loss of Dlx3 function resulted in a specific reduction of Sox9 otic expression domain, suggesting that these factors may act in the same regulatory pathway during otic placode formation. However, the Dlx3-mo-injected embryos appeared to form a normal otic vesicle. This could be explained by the fact that Dlx3-mo generate only an incomplete loss of Sox9 and that a Dlx3-independent pathway may also be involved in the regulation of Sox9 otic expression. This is in agreement with recent work that has implicated the zebrafish Dlx3 homolog, Dlx3b, in otic placode formation (a function partially shared with the linked and co-expressed Dlx4b gene) (Solomon and Fritz, 2002) and demonstrated the existence of a Dlx3-dependent and Dlx3-independent regulation of Sox9a (the zebrafish Sox9 homolog) expression during otic placode formation (Liu et al., 2003). Moreover, the observation that Pax8 and Tbx2 are not affected in Dlx3-depleted embryos is consistent with the view that Dlx3 is required for maintenance of Sox9 expression following Sox9 initial function in otic placode specification.

Experiments in amphibian have established that otic placode induction is a multiple step process that requires signals derived from the mesoderm and the neuroectoderm (Jacobson, 1966; Gallagher et al., 1996) (reviewed by Noramly and Grainger, 2002). More recent studies in chick, zebrafish and mouse have identified some of these signals. There is evidence that molecules belonging to the fibroblast growth factor (Fgf) family, expressed in the paraxial mesoderm (Fgf10 and Fgf19) and the hindbrain (Fgf3 and Fgf8) are involved in otic vesicle induction and patterning (Vendrell et al., 2000; Ladher et al., 2000; Phillips et al., 2001; Leger and Brand, 2002; Liu et al., 2003; Wright and Mansour, 2003; Alvarez et al., 2003). Additionally, work in the chick embryo suggests that molecules of the Wnt family are also involved in this process. Wnt8c, which is expressed in the hindbrain, appears to be required in combination with a Fgf signal (Fgf19) to generate the otic placode from undifferentiated ectoderm (Ladher et al., 2000). In *Xenopus* the otic expression of Sox9 appears to be under the dual control of Fgf and Wnt signaling, as interference with either signaling pathway blocks Sox9 otic expression. However, because Fgf and Wnt signaling have also been implicated in mesoderm and neural patterning, the loss of Sox9

expression in the otic placode/vesicle may reflect a disruption of the putative inducing tissues rather than the inducing molecules. Moreover, the observation that embryos with compromised Fgf or Wnt signaling form an otic vesicle of reduced size may suggest: (1) that only an incomplete inhibition of these pathways was attainable in our experimental conditions; (2) that both pathways are required concurrently; or (3) that other factors are also required for the induction of the otic placode in *Xenopus*.

Although expression of a dominant-negative Fgf receptor (Amaya et al., 1991) and overexpression of GSK3β (He et al., 1994) are likely to block a broad range of Fgf and Wnt family members, respectively, these experiments suggest the existence of an endogenous Fgf and Wnt signal implicated in Sox9 expression in the otic palcode. Wnt8 is expressed in the paraxial mesoderm and detected at the time of otic placode specification (Christian and Moon, 1993) and could therefore represent this endogenous Wnt signal involved in otic placode induction in *Xenopus*. Fgf3 is also a good candidate inducer as it is detected in the prospective hindbrain at the late gastrula stage (Lombardo et al., 1998). Additionally, Fgf2 has been shown to promote formation of supernumerary otic vesicles when applied ectopically (Lombardo and Slack, 1998), and therefore could be involved in initiating Sox9 expression in the otic placode. The identification of these ligands will be key in understanding the contribution of these two signaling pathways to the induction of the otic placode in *Xenopus*.

Sox9 depletion resulted in a severe loss of early (Pax8 and Tbx2) and late (Tbx2, Wnt3a, Otx2 and Bmp4) otic markers. This phenotype is also characterized by the inability of the otic placodal ectoderm to thicken and generate an otic vesicle, suggesting that Sox9 is required for inner ear development in *Xenopus*. Overexpression of *Xenopus* or mouse Sox9 in the ventral ectoderm did not lead to ectopic Pax8 expression or formation of supernumerary otic vesicles (not shown). This result indicates that although Sox9 is required it is not sufficient to initiate formation of the otic placode and argues for the requirement of additional factors.

To further define the window of time during which Sox9 is functioning during otic placode formation, we generated a hormone inducible inhibitory Sox9 construct (Sox9ΔC-GR), to produce a temporal inactivation of Sox9 function. Inactivation of Sox9 during gastrulation (stage 11), and prior to the neural plate stage (stage 15), prevented Pax8 and Tbx2 otic expression. This observation indicates that a Sox9-dependent pathway is required for specification of the otic placode, and that the inductive events involved are taking place prior to the neural plate stage. This is consistent with transplantation experiments in *Xenopus* showing that some level of placodal specification has already occurred by early neurula stage (Jacobson, 1966; Gallagher et al., 1996). Conversely, inactivation Sox9 at the neurula stage (stage 15) and beyond (stage 20), which bypass the early requirement for Sox9, had no effect on the development of the otic placode and the patterning of the otic vesicle. This result is of importance as it implies that Sox9 is strictly functioning during the initial stages of specification of the otic placode.

Campomelic dysplasia (OMIM #114290) is a severe skeletal dysmorphology syndrome caused by haploinsufficiency of Sox9 (Foster et al., 1994; Wagner et al., 1994; McDowall et al., 1999; Wunderle et al., 1998; Olney et al., 1999; Preiss et

al., 2001). Individuals affected by this condition usually die shortly after birth because of respiratory distress; therefore, it has been difficult to evaluate the auditory capability of these individuals. However, reports of rare individuals that survived through adolescence (Houston et al., 1983) and adulthood (Savarirayan et al., 2003) indicate that they are affected with sensorineural deafness. Our results in *Xenopus* predict that hearing loss associated with campomelic dysplasia is likely to be the result of an early defect in the specification of the otic placode. However, the lack of information on the nature of the inner ear defects associated with this pathology in human, and in a mouse model of the disease (Bi et al., 2001), makes it difficult, at this time, to fully assess this prediction.

The strict requirement of Sox9 for specification of the inner ear in *Xenopus* raises the possibility that other Sox family members might be more directly involved in the differentiation and the patterning of the otic vesicle. For example, Sox10, whose expression and function in the developing neural crest has been well studied in several species (Southard-Smith et al., 1998; Kapur, 1999; Dutton et al., 2001; Aoki et al., 2003), is also expressed in the entire epithelium of the otic vesicle in human, mouse, chick, frog and fish (Bondurand et al., 1998; Southard-Smith et al., 1998; Watanabe et al., 2000; Cheng et al., 2000; Dutton et al., 2001; Aoki et al., 2003). Sox10 heterozygous mutations have been identified in individuals who suffer from Waardenburg-Shah (WS4) syndrome (OMIM#277580). These individuals exhibit aganglionic megacolon, hypopigmentation and sensorineural deafness (Pingault et al., 1998; Southard-Smith et al., 1999). The phenotype associated with this pathology indicates an important role for Sox10 in neural crest formation but also in the development of the inner ear. In *Xenopus*, Sox9 otic expression precedes that of Sox10, which is detected only when the otic placode starts to invaginate into a vesicle (Spokony et al., 2002; Aoki et al., 2003). This observation suggests that Sox10 may have function during the later phase of differentiation and patterning of the otic vesicle rather than its specification. A comparative analysis of the inner ear defects of individuals affected by campomelic dysplasia and Waardenburg-Shah syndromes should provide important information on the respective contribution of these two Sox family members to the development of the vertebrate inner ear.

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