

Original Article

Sox9 regulates development of neural crest and otic placode in a time- and dose-dependent fashion

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Neural crest and placodes share a number of important features, pointing to a possible common evolutionary origin. They both arise from the neural plate border, which is the boundary between the non-neural ectoderm and neural plate. The transcription factor Sox9 has been implicated in neural crest and otic placode induction in several species. To investigate the differential regulation of neural crest and otic placode induction by Sox9, a gain of function assay was performed using a hormone-inducible version of the Sox9 construct at different doses and time periods. Sox9 was expressed in both neural crest and otic placode cell populations in the same stage embryos by in situ hybridization. Using a gain of function approach, increased expression of neural crest marker (Snail2) and otic placode marker (Pax8) in Sox9-overexpressed embryos was observed. Higher dose of Sox9 reduced or eliminated both neural crest and placode cells in the embryos. Interestingly, otic placodes cells were more strongly affected as compared to neural crest cells. So, optimal dosage and timing of Sox9 expression are important for the development of the neural crest and otic placode. The development of the neural crest and otic placode are affected by Sox9 in a time- and dose-dependent manner.

Key words: Sox9, neural crest, otic placode, development, *Xenopus*

Introduction

Vertebrate ectoderm is differentiated into neural plate, neural plate border and non-neuronal ectoderm after gastrulation. The neural plate border, which consists of neural crest cells and cranial placode cells, is located between the non-neural ectoderm and the neural plate. The neural crest cells and the neural placodes delaminate from the epithelial structure from which they originate, and they have the ability to differentiate into a large ar-

ray of cell types including sensory neurons, neuroendocrine cells, glia and supporting cells [1]. Neural crest and placodes share a number of important features pointing to a possible common evolutionary origin [2]. It has been suggested that neural crest and placodes may have evolved from a common ancestral population present in vertebrate ancestors [3].

The development of neural crest and placode involves morphological changes of cells, and both neural crest and placodes develop from populations of cells near the border of the neural plate [4]. The neural crest is a multipotent population of cells that is characteristic of vertebrate embryos posterior to the diencephalon. Several types of placodes can be distinguished at the neural border: adeno-hypophysial placode, olfactory placode, lens placode, profundal placode, trigeminal placode, a series of epibranchial placodes contributing to the distal ganglia of the facial, glossopharyngeal, and vagal nerves, hypobranchial placodes, a series of lateral line placodes giving rise to the lateral line ganglia and lateral line organs, and the otic placode [5-7]. Among the cranial placodes, the otic placode is particularly remarkable because the inner ear and the vestibulocochlear ganglion develop from it. The induction and development of the otic placode has long been a popular subject of experimental embryology studies because it is readily accessible and undergoes such a complex morphogenesis [8].

The Sox proteins constitute a large array of transcription factors [9]. Among the Sox family members, Sox9 regulate the chondrogenesis in mouse embryos [10, 11]. Mutation of Sox9 results in Campomelic Dysplasia and severe skeletal malformations [12-14]. Sox9 is an essential component for the regulation of neural crest formation and a key regulator of inner ear specification in *Xenopus* [15]. Morpholino mediated knockdown of Sox9 generated embryos exhibiting a specific loss of early neu-

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ral crest markers [16]. Wnt signaling regulates the induction of neural crest progenitors depending on the functions of Sox9 as Wnt mediated neural crest induction is inhibited in the context of Sox9 depleted embryos, which suggests the function of Sox9 in neural crest specification [17]. Overexpression studies on Sox9 cause ectopic expression of several of early neural crest markers, consistent with an early neural crest formation [18]. Depletion of Sox9 protein in *Xenopus* whole embryos using morpholino antisense oligonucleotides causes a dramatic loss of the early otic placode markers [15].

Despite the data available regarding the expression and functional analyses of Sox9 in the induction of neural crest and otic placode, there have been no dose dependent regulations of Sox9 during *Xenopus* embryogenesis. Here it is reported the regulation of Sox9 in the development of neural crest and otic placode in a time and dose dependent fashion.

Materials and Methods

Embryo collection and manipulation

Xenopus laevis were handled in accordance with animal welfare regulations of Institutional Animal Care and Use Committees (IACUC), Chonbuk National University Laboratory Animal Centre, South Korea. Embryos were obtained from adult *Xenopus laevis* by standard hormone-induced egg laying and artificial fertilization. All efforts were made to minimize the discomfort of animal used.

Construct

Hormone-inducible constructs were generated by subcloning the coding region of *Sox9* gene into pCS2 + GR [19]. Fusion constructs subcloned into pCS2+, referred as Sox9-GR, were sequenced, and the corresponding protein was monitored using an *in vitro* transcription/translation coupled rabbit reticulocyte lysate system (Promega, Madison, WI). Sox9-GR mRNA was synthesized *in vitro* by using Message Machine kit (Ambion, Austin, TX).

Embryo injections and dexamethasone treatment

Synthetic Sox9-GR mRNA was injected into the animal pole of one blastomere at the two-cell stage. Embryos were also co-injected with β -galactosidase mRNA (β -gal, 1 ng). Sox9-GR injected embryos were cultured in 0.1 \times normal amphibian medium (NAM) containing 10 μ M dexamethasone from stage 14 or stage 17. Siblings injected with these constructs and cultured in the absence of dexamethasone were used as control.

Lineage tracing and In situ hybridization

Embryos at the appropriate stage were fixed in MEMFA and successively processed for Red Gal staining (Re-

search Organics) to identify the injected side and *in situ* hybridization. Antisense DIG-labeled probes (Genius kit, Roche) were synthesized using template cDNA encoding *Pax8* [20] and *Snail2* [21] for *in situ* hybridization. Whole-mount *in situ* hybridization was performed as previously described [22]. For *in situ* hybridization on sections, embryos were fixed in MEMFA for 1 hr and embedded in Paraplast. Sections (12 μ m) were then cut on a rotary microtome and hybridized with the appropriate probes as described previously [23]. Sections were then briefly counterstained with eosin.

Results

Sox9 is expressed in the neural crest and otic placode at neurula stage

Using whole mount *in situ* hybridization the expression patterns of Sox9 were analyzed in developing *Xenopus*. At neurula stage, Sox9 was expressed both in the neural crest and otic placode domain (Fig. 1A). Tissue sections of stage 15 embryos highlighted Sox9 expression in the neural crest as well as in the prospective otic placode that appears to be confined to the sensory layer of the ectoderm (Fig. 1B). By the tailbud stage, the diffuse expression of Sox9 in the presumptive otic placode was more confined to the otic vesicle and branch arches (Fig. 1C). To determine the tissue specific expression analysis of Sox9 gene, whole mount *in situ* hybridization for both *Snail2* and *pax8* was performed in staged matched embryos. Both Sox9 and *Snail2* were expressed in the devel-

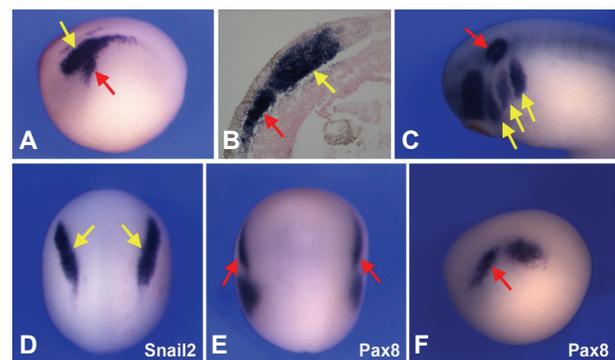


Fig. 1. Normal expression of Sox9. (A) At neurula stage Sox9 is expressed neural crest and otic placode. Left to the anterior. (B) Cross-section through the neural crest and prospective otic placode of a stage 14 embryo, Sox9 is detected in the neural crest and prospective otic placode. (C) At the tailbud stage, Sox9 persists its expression in the otic vesicle and branchial arches which gives rise from the neural crest cells. (D) Expression of *Snail2* in the neural crest cells. (E) For comparison, *Pax8* expression at the neurula stage is shown. *Pax8* is detected in the prospective otic placode (red arrow). Dorsal view, anterior towards the top. (F) Lateral view of *Pax8* expression, anterior towards the left. Otic placode (red arrow), neural crest cells and its derivatives (yellow arrow).

opening neural crest (Figs. 1A and 1D), and Sox9 shared a common expression domain with Pax8 in the prospective otic placode (Figs. 1A, 1E and 1F).

Development of neural crest and otic placode is regulated by Sox9 in time and dose dependent manner

To evaluate the role of Sox9 in the induction of neural crest and otic placode, a hormone-inducible version of Sox9 was injected in the two-cell stage embryos in which Sox9 was fused to the hormone-binding domain of human glucocorticoid receptor (Sox9-GR). The activity of this fusion protein can be regulated by addition of dexamethasone to the embryo culture medium [24]. Embryos injected with 0.05 ng, 0.2 ng or 0.5 ng of Sox9-GR mRNA were treated with dexamethasone at late gastrula stage (stage 10.5) or early neurula stage (stage 14) and analyzed for gene expression at late neurula (stage 17) or early tailbud (stage 22) stage. Overexpression of Sox9 (0.05 ng) at the gastrula stage before induction of neural

crest and otic placode resulted in an expansion of the both neural crest specific marker gene *Snail2* and otic placode marker gene *Pax8* expression at late neurula stage and at early tailbud stage, respectively (Figs. 2B and 2C). Sox9 overexpression (0.2 ng) at gastrula stage was consistent with the expression at low (0.05 ng) dose but at higher dose (0.5 ng) expression of both *Snail2* and *Pax8* was reduced or lost (Figs. 2B and 2C).

Over expression of Sox9 at gastrula stage before induction of neural crest and otic placode showed an interesting change. At stage 17, unilateral injection of low dose of Sox9-GR mRNA (0.05 ng) resulted in almost normal expression (89.4%, n=47) of *Snail2* in neural crest, whereas injection of the mid-dose level of (0.2 ng) Sox9-GR resulted in a marked expansion of *Snail2* expression in more than 60% (n=36) of the embryos analyzed. But higher dose (0.5 ng) of Sox9 overexpression showed a different scenario that the expression of *Snail2* was mostly reduced (69.2%, n=39) (Fig. 2C). In case of injection of Sox9-GR mRNA at lower dose (0.05 ng), the otic

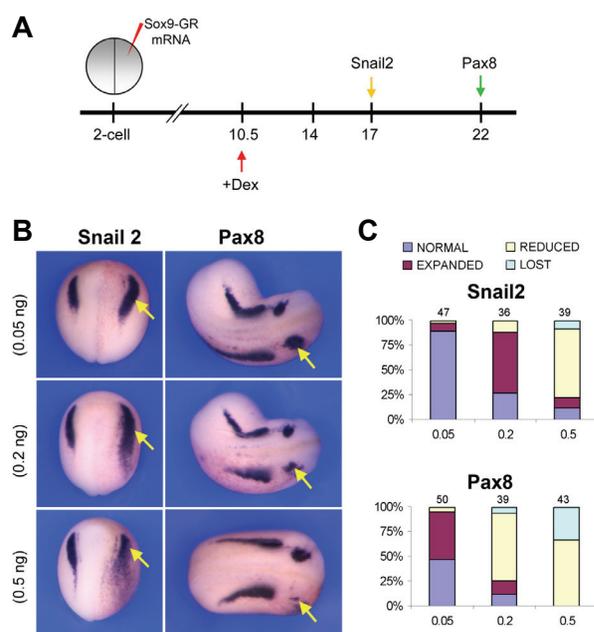


Fig. 2. Overexpression of Sox9 before induction of neural crest and otic placode. (A) Two-cell stage embryos were injected with Sox9-GR mRNA on blastomere. Embryos were subsequently incubated with dexamethasone (+Dex) at stage 10.5 and fixed at stage 17 and stage 22 to detect *Snail2* and *Pax8* expression by whole-mount *in situ* hybridization, respectively. (B) Expression of *Snail2* (neural crest specific marker) and *Pax8* (otic placode marker) by whole mount *in situ* hybridization in Sox9-GR mRNA injected embryos (Sox9-GR; 0.05 ng, 0.2 ng, and 0.5 ng). All panels are dorsal views. Yellow arrow indicates expression of *Snail2* or *Pax8* of the Sox9-GR injected side. RNA encoding the lineage tracer β galactosidase was co-injected to identify the injected side (red staining). (C) Graphical representation of whole-mount *in situ* hybridization results. The number on the top of each bar indicates the number of embryo analyzed.

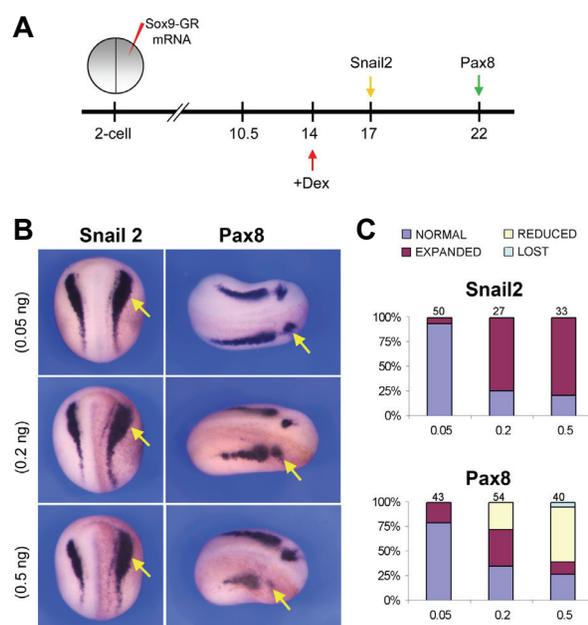


Fig. 3. Overexpression of Sox9 after induction of neural crest and otic placode. (A) Embryos at the two-cell stage were injected with Sox9-GR mRNA on blastomere. Embryos were subsequently incubated with dexamethasone (+Dex) at stage 14 and fixed at stage 17 and stage 22 for the detection of whole-mount *in situ* hybridization expression of *Snail2* and *Pax8*, respectively. (B) Representative case of *Snail2* and *Pax8* whole-mount *in situ* hybridization of Sox9-GR injected embryos (Sox9-GR; 0.05 ng, 0.2 ng, and 0.5 ng). All panels are dorsal views. Yellow arrow indicates expression of *Snail2* or *Pax8* of the Sox9-GR mRNA injected side. RNA encoding the lineage tracer β -galactosidase was co-injected to identify the injected side (red staining). (C) Quantification of whole-mount *in situ* hybridization results. The number on the top of each bar indicates the number of embryo analyzed.

placode marker Pax8 was expanded in 48% (n=50) of the embryos but at the mid-dose level (0.2 ng) of Sox9-GR, Pax8 expression was reduced in 69.2% (n=39) of the embryos at stage 22. At higher dose (0.5 ng), Pax8 expression was mostly reduced or lost in Sox9-overexpressed embryos (67.4%, n=43 and 32.6%, n=43, respectively) (Fig. 2C).

After induction of neural crest cells and otic placode the expression of Snail2 and Pax8 showed a different level of expression in the embryos analyzed. In this case, injection of 0.05 ng Sox9-GR mRNA resulted in almost normal expression of Snail2 in 94% (n=50) of the embryos whereas at 0.2 ng dose level Snail2 expression was mostly expanded in 74.1% (n=27) of the embryos and at the higher dose level consistent with the expression of 0.2 ng dose level (78.8%, n=33) (Figs. 3B and 3C). Pax8 showed almost normal expression in 79.1% (n=43) of the embryos injected with lower dose (0.05 ng) of Sox9-GR but injection of 0.2 ng Sox9-GR resulted in expansion of Pax8 expression in 37% (n=54) of the embryos. 55% of higher dose Sox9-GR injected embryos (n=40) exhibited a reduction of Pax8 expression in the otic placode (Figs. 3B and 3C).

Discussion

Head skeleton and sensory ganglia originate from two embryonic structures: the neural crest and the cranial placodes. Neural crest arises from the entire length of the neural tube starting from a region posterior to the prospective diencephalon and will develop a broad cell types including pigment cells, cartilage and smooth muscle cells. Craniofacial sensory organs arise from placodal structures located at the periphery of the neural plate [1, 25]. The inner ear is derived from a simple patch of thickened ectoderm, the otic placode that arises next to the posterior hindbrain [26-29]. Previous studies suggest the role of hindbrain in otic placode induction and, members of the fibroblast growth factors (Fgf) family play a crucial role in this induction in several species [30-33]. Fgf originated paraxial mesoderm is also required for the induction of neural crest [34]. Wnt signaling has also been proposed to participate in otic placode induction and early neural crest induction. Both signals control and regulate the signaling in a dose dependent manner. In *Xenopus* Sox9 is a downstream of Wnt and Fgf, and both of them control Sox9 regulations regarding the development of neural crest and otic placode [33-35].

In this study it was found that at early neurula stage, Sox9 was highly expressed in the migrating neural crest and otic placode domain which were located at the periphery of the neural plate. By the tailbud stage, the expression was confined to the otic vesicle and branchial arches which arose from the neural crest cells. This result

is consistent with Sox9 requirements for the maintenance of progenitors in the otic vesicle [36]. Sox proteins are known to regulate their target genes through interaction with cell specific partner factors [37]. The expansion of the neural crest and otic placode in Sox9-GR injected embryos could reflect the inability of the embryo to regulate the proper level of neural crest marker gene Snail2 and the otic placode specific gene Pax8.

Though it is not clear how Sox9 controls the positional genes that lead to neural crest and otic placode formation, Sox9 controls the development of dorsal and ventral part of otic vesicle in a dose dependent manner [36]. Sox9 differently interferes the expression of neural crest and otic placode specific genes. At the same dose level of Sox9, the expression of otic placode specific gene more severely affected than neural crest marker gene. In the present study, in low dose of Sox9-GR mRNA injected embryos, Snail2 expression was mostly normal and reduced or expanded with the increasing dose level. But too higher dose may reduce or lose the expression of neural crest marker genes.

Sox9 overexpression affected the expression of neural crest marker and otic placode marker in both time and dose dependently. The expression of neural crest marker gene, Snail2 and otic placode marker gene, Pax8 showed a different phenotype when Sox9 was overexpressed either before induction of neural crest and otic placode (stage 10.5) and after induction but before specification (stage 14). After cellular induction lower dose of Sox9-GR mRNA injection resulted in almost normal expression of Snail2 whereas Pax8 was expanded in more than 50% of the embryos. It may be related with the timing of induction and differentiation of the otic placode. Placodes derive from a common pre-placodal domain consisting of a narrow band of ectoderm that encompasses the anterior neural plate that is established at the end of gastrulation [1, 25, 38, 39]. Cranial placodes form in two steps. The first step is the acquisition of a general placodal state (pan-placodal domain) in the ectodermal domain adjacent to the neural ectoderm, with the second step being the determination of the identity of each placode by local inducing signals [40]. Due to a timing of induction of neural crest, Snail2 was not affected acutely whereas Pax8 was expanded due to the differentiation and specification of otic placode. And in higher dose, neural crest was largely expanded, but Pax8 was mostly reduced, which suggested that Sox9 controlled the genes expressed in the neural crest and otic placode domain in a time and dose dependent manner.

Therefore, it should be proposed that Sox9 is an important regulator for the maintenance of neural crest and otic placode formation and mediation of cell fate where it controls the signaling pathway in a time and dose dependent fashion.

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References

- Baker CV, Bronner-Fraser M. Vertebrate cranial placodes I. Embryonic induction. *Dev Biol* 2001;232:1-61.
- A R, Tyson JR, Stirling CJ. A novel subfamily of Hsp70s in the endoplasmic reticulum. *Trends Cell Biol* 1997;7:277-282.
- Baker CV, Bronner-Fraser M. The origins of the neural crest. Part II: an evolutionary perspective. *Mech Dev* 1997;69:13-29.
- Schlosser G. Induction and specification of cranial placodes. *Dev Biol* 2006;294:303-351.
- Schlosser G, Kintner C, Northcutt RG. Loss of ectodermal competence for lateral line placode formation in the direct developing frog *Eleutherodactylus coqui*. *Dev Biol* 1999;213:354-369.
- Schlosser G, Northcutt RG. Development of neurogenic placodes in *Xenopus laevis*. *J Comp Neurol* 2000;418:121-146.
- Schlosser G. Hypobranchial placodes in *Xenopus laevis* give rise to hypobranchial ganglia, a novel type of cranial ganglia. *Cell Tissue Res* 2003;312:21-29.
- Phillips BT, Storch EM, Lekven AC, Riley BB. A direct role for Fgf but not Wnt in otic placode induction. *Development* 2004;131:923-931.
- Wegner M. From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res* 1999;27:1409-1420.
- Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrughe B. Sox9 is required for cartilage formation. *Nat Genet* 1999;22:85-89.
- Bi W, Huang W, Whitworth DJ, Deng JM, Zhang Z, Behringer RR, de Crombrughe B. Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization. *Proc Natl Acad Sci USA* 2001;98:6698-6703.
- Houston CS, Opitz JM, Spranger JW, Macpherson RI, Reed MH, Gilbert EF, Herrmann J, Schinzel A. The campomelic syndrome: review, report of 17 cases, and follow-up on the currently 17-year-old boy first reported by Maroteaux et al in 1971. *Am J Med Genet* 1983;15:3-28.
- Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M, Weissenbach J, Mansour S, Young ID, Goodfellow PN, Brook JD, Schafer AJ. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature* 1994;372:525-530.
- Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantés J, Bricarelli FD, Keutel J, Hustert E, Wolf U, Tommerup N, Schempp W, Scherer G. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell* 1994;79:1111-1120.
- Saint-Germain N, Lee YH, Zhang Y, Sargent TD, Saint-Jeannet JP. Specification of the otic placode depends on Sox9 function in *Xenopus*. *Development* 2004;131:1755-1763.
- Spokony RF, Aoki Y, Saint-Germain N, Magner-Fink E, Saint-Jeannet JP. The transcription factor Sox9 is required for cranial neural crest development in *Xenopus*. *Development* 2002;129:421-432.
- Lee YH, Aoki Y, Hong CS, Saint-Germain N, Credidio C, Saint-Jeannet JP. Early requirement of the transcriptional activator Sox9 for neural crest specification in *Xenopus*. *Dev Biol* 2004;275:93-103.
- Yan YL, Willoughby J, Liu D, Crump JG, Wilson C, Miller CT, Singer A, Kimmel C, Westerfield M, Postlethwait JH. A pair of Sox: distinct and overlapping functions of zebrafish sox9 co-orthologs in craniofacial and pectoral fin development. *Development* 2005;132:1069-1083.
- Tada M, O'Reilly MA, Smith JC. Analysis of competence and of Brachyury autoinduction by use of hormone-inducible Xbra. *Development* 1997;124:2225-2234.
- Heller N, Brandli AW. *Xenopus* Pax-2/5/8 orthologues: novel insights into Pax gene evolution and identification of Pax-8 as the earliest marker for otic and pronephric cell lineages. *Dev Genet* 1999;24:208-219.
- Mayor R, Morgan R, Sargent MG. Induction of the prospective neural crest of *Xenopus*. *Development* 1995;121:767-777.
- Harland RM. In situ hybridization: an improved whole-mount method for *Xenopus* embryos. *Methods Cell Biol* 1991;36:685-695.
- Henry GL, Brivanlou IH, Kessler DS, Hemmati-Brivanlou A, Melton DA. TGF-beta signals and a pattern in *Xenopus laevis* endodermal development. *Development* 1996;122:1007-1015.
- Kolm PJ, Sive HL. Regulation of the *Xenopus* labial homeodomain genes, HoxA1 and HoxD1: activation by retinoids and peptide growth factors. *Dev Biol* 1995;167:34-49.
- Brugmann SA, Moody SA. Induction and specification of the vertebrate ectodermal placodes: precursors of the cranial sensory organs. *Biol Cell* 2005;97:303-319.
- Barald KF, Kelley MW. From placode to polarization: new tunes in inner ear development. *Development*

- 2004;131:4119-4130.
27. Brown ST, Martin K, Groves AK. Molecular basis of inner ear induction. *Curr Top Dev Biol* 2003;57:115-149.
 28. Riley BB, Phillips BT. Ringing in the new ear: resolution of cell interactions in otic development. *Dev Biol* 2003;261:289-312.
 29. Torres M, Giraldez F. The development of the vertebrate inner ear. *Mech Dev* 1998;71:5-21.
 30. Ladher RK, Wright TJ, Moon AM, Mansour SL, Schoenwolf GC. FGF8 initiates inner ear induction in chick and mouse. *Genes Dev* 2005;19:603-613.
 31. Leger S, Brand M. Fgf8 and Fgf3 are required for zebrafish ear placode induction, maintenance and inner ear patterning. *Mech Dev* 2002;119:91-108.
 32. Liu D, Chu H, Maves L, Yan YL, Morcos PA, Postlethwait JH, Westerfield M. Fgf3 and Fgf8 dependent and independent transcription factors are required for otic placode specification. *Development* 2003;130:2213-2224.
 33. Lombardo A, Isaacs HV, Slack JM. Expression and functions of FGF-3 in *Xenopus* development. *Int J Dev Biol* 1998;42:1101-1107.
 34. Monsoro-Burq AH, Fletcher RB, Harland RM. Neural crest induction by paraxial mesoderm in *Xenopus* embryos requires FGF signals. *Development* 2003;130:3111-3124.
 35. Ladher RK, Anakwe KU, Gurney AL, Schoenwolf GC, Francis-West PH. Identification of synergistic signals initiating inner ear development. *Science* 2000;290:1965-1967.
 36. Park BY, Saint-Jeannet JP. Long-term consequences of Sox9 depletion on inner ear development. *Dev Dyn* 2010;239:1102-1112.
 37. Kamachi Y, Uchikawa M, Kondoh H. Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet* 2000;16:182-187.
 38. Streit A. Early development of the cranial sensory nervous system: from a common field to individual placodes. *Dev Biol* 2004;276:1-15.
 39. Bailey AP, Streit A. Sensory organs: making and breaking the pre-placodal region. *Curr Top Dev Biol* 2006;72:167-204.
 40. Ohyama T, Groves AK, Martin K. The first steps towards hearing: mechanisms of otic placode induction. *Int J Dev Biol* 2007;51:463-472.