

Changes in retinoic acid signaling alter otic patterning

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Retinoic acid (RA) has pleiotropic functions during embryogenesis. In zebrafish, increasing or blocking RA signaling results in enlarged or reduced otic vesicles, respectively. Here we elucidate the mechanisms that underlie these changes and show that they have origins in different tissues. Excess RA leads to ectopic *foxi1* expression throughout the entire preplacodal domain. *Foxi1* provides competence to adopt an otic fate. Subsequently, *pax8*, the expression of which depends upon *Foxi1* and *Fgf*, is also expressed throughout the preplacodal domain. By contrast, loss of RA signaling does not affect *foxi1* expression or otic competence, but instead results in delayed onset of *fgf3* expression and impaired otic induction. *fgf8* mutants depleted of RA signaling produce few otic cells, and these cells fail to form a vesicle, indicating that *Fgf8* is the primary factor responsible for otic induction in RA-depleted embryos. Otic induction is rescued by *fgf8* overexpression in RA-depleted embryos, although otic vesicles never achieve a normal size, suggesting that an additional factor is required to maintain otic fate. *fgf3; tcf2* double mutants form otic vesicles similar to RA-signaling-depleted embryos, suggesting a signal from rhombomere 5–6 may also be required for otic fate maintenance. We show that rhombomere 5 *wnt8b* expression is absent in both RA-signaling-depleted embryos and in *fgf3; tcf2* double mutants, and inactivation of *wnt8b* in *fgf3* mutants by morpholino injection results in small otic vesicles, similar to RA depletion in wild type. Thus, excess RA expands otic competence, whereas the loss of RA impairs the expression of *fgf3* and *wnt8b* in the hindbrain, compromising the induction and maintenance of otic fate.

KEY WORDS: Competence, *dlx3b*, *Danio rerio*, *fgf3*, *fgf8*, *foxi1*, Inner ear, Morpholino, Otic induction, Otic placode, Retinoic acid, Zebrafish

INTRODUCTION

The vertebrate inner ear is the sensory organ that provides auditory and vestibular functions. It develops from a transient ectodermal thickening, the otic placode, visible on either side of the developing hindbrain. Depending on the species, the placode invaginates or cavitates to form the otic vesicle, also known as the otocyst, an epithelial structure with sharply defined borders. Subsequently, the otocyst gives rise to all structures of the inner ear including the membranous labyrinth and neurons of the statoacoustic ganglion (Noden and van de Water, 1986; Couly et al., 1993; Fritzsche et al., 1997; Whitfield et al., 2002; Barald and Kelley, 2004).

Previous studies support the primacy of fibroblast growth factors (Fgfs) in the induction of the otic placode. Various *Fgf* family members from various sources regulate otic induction in different species, with only hindbrain-derived *Fgf3* playing a conserved role (reviewed in Fritzsche et al., 1997; Torres and Giráldez, 1998; Whitfield et al., 2002; Brown et al., 2003). In zebrafish, *Fgf3* and *Fgf8* have been implicated to have overlapping functions; loss of both *fgf3* and *fgf8* together results in near or total ablation of otic tissue (Phillips et al., 2001; Maroon et al., 2002; Léger and Brand, 2002). In mouse, *Fgf3* and *Fgf10* act as redundant signals during otic induction (Wright and Mansour, 2003; Alvarez et al., 2003): *Fgf3* is expressed in the hindbrain abutting the preotic domain, whereas *Fgf10* is expressed in the mesoderm beneath it, and loss of both *Fgf3* and *Fgf10* results in the complete ablation of otic development (Wright and Mansour, 2003; Alvarez et al., 2003). Furthermore, *Fgf8* has been shown to play a crucial role upstream of the *Fgf* signaling cascade required for otic induction in this species (Ladher

et al., 2005). In chick, *Fgf3*, *Fgf8* and *Fgf19* and, in amphibians, *Fgf2* and *Fgf3*, have been implicated in otic induction (Mahmood et al., 1995; Ladher et al., 2000; Ladher et al., 2005; Song and Slack, 1994; Lombardo et al., 1998).

Recently, studies have shown that *Pax2a* and *Pax8* are the main effectors downstream of *Fgf*-signaling and that cells need to express *Foxi1* and *Dlx3b* transcription factors to be competent to respond to *Fgf* signaling in zebrafish (Hans et al., 2004; Solomon et al., 2004). The expression of *foxi1* is restricted to bilateral domains, including the preotic domain at late gastrula stages, and disruption of *foxi1* expression leads to severe defects in otic placode formation and highly variable ear phenotypes (Thisse et al., 2005; Solomon et al., 2003; Nissen et al., 2003). The homeobox gene, *dlx3b*, is co-expressed with *dlx4b* in late gastrula stage embryos in a stripe corresponding to cells of the future neural plate border, and knockdown of *dlx3b* and *dlx4b* together causes a severe loss of otic and olfactory tissues (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997; Kudoh et al., 2001; Solomon and Fritz, 2002; Liu et al., 2003). Loss of both *Dlx3b* and *Foxi1* ablates all indications of otic induction even in the presence of a fully functional or over-activated *Fgf* signaling pathway (Hans et al., 2004; Solomon et al., 2004; Hans et al., 2007).

Retinoic acid (RA), a derivative of vitamin A, is required for proper embryonic development. Embryos deficient in RA signaling show defects in the circulatory system, limbs, trunk and hematopoietic system (reviewed by Maden, 2002). RA plays a crucial role in hindbrain patterning and rhombomere (r) identity, and the hindbrain is known to regulate otic development (reviewed by Gavalas and Krumlauf, 2000; Romand, 2003). In amniote embryos, RA is required in a concentration- and time-dependent manner for the development of the posterior hindbrain, particularly r5–r7, and compromised RA signaling leads to an expansion of anterior hindbrain at the expense of posterior hindbrain (Dupé et al., 1999; Dupé and Lumsden, 2001). Complete absence of RA signaling leads to a complete loss of r5–r7 accompanied by an expansion of r3–r4,

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as observed in mouse embryos mutant for *Aldh1a2* (also known as *Raldh2* – Mouse Genome Informatics). *Aldh1a2* is an aldehyde dehydrogenase that is responsible for the majority of RA production in the early embryo (Niederreither et al., 1999; Niederreither et al., 2000). Mutations in *aldh1a2* in zebrafish are less profound and show only a loss of r7 accompanied by a slight expansion of r5 and r6 similar to weak vitamin A deficiency syndrome (VAD) in amniote embryos (Begemann et al., 2001; Grandel et al., 2002; Maves and Kimmel, 2005). However, a loss of r5-r7 accompanied by an expansion of r3 and r4 is observed after knockdown of RA signaling by pharmacological treatment, suggesting that RA is produced by something in addition to *Aldh1a2* (Grandel et al., 2002; Maves and Kimmel, 2005). Treatment of vertebrate embryos with excess RA posteriorizes the anterior neural plate with the transformation of r2-r3 to r4-r5 identity and expansion of posterior hindbrain at the expense of presumptive fore- and mid-brain structures (Marshall et al., 1992; Kudoh et al., 2002).

Otic defects generated by changes in RA signaling are considered mostly secondary consequences due to changes in Fgf signaling, because hindbrain patterning is disturbed in RA gain- and loss-of-function studies. In embryos with no RA signaling, such as mouse *Aldh1a2* mutants, expression of *Fgf3*, which is specifically expressed in the presumptive r5-r6 in wild-type embryos, is weak and not properly restricted, and otocysts are hypoplastic and abnormally distant from the hindbrain (Niederreither et al., 1999). The same otic phenotype can be observed in zebrafish after knockdown of RA by pharmacological treatment and it has been suggested, but not yet shown, that RA is required for normal *fgf3* expression in the presumptive r4 and for the induction of the otic placode (Perz-Edwards et al., 2001; Whitfield et al., 2002). A moderate loss of RA signaling, such as in weak quail or rat VAD embryos, however, leads to the formation of supernumerary otic vesicles caudal to the main otocyst, which correlates with the expansion of posterior hindbrain and, in particular, with the caudal expansion of *Fgf3* expression (White et al., 2000; Kil et al., 2005). In zebrafish, the opposite result has been reported; excess, rather than reduced, RA results in the formation of supernumerary and ectopic otic vesicles in an Fgf-dependent manner (Phillips et al., 2001). In zebrafish embryos treated with teratogenic doses (1 μ M) of RA, hindbrain expression domains of *fgf3* and *fgf8* are greatly expanded into the anterior neural plate, inducing preotic *pax8* expression surrounding the anterior neural plate. Inactivation of *Fgf3* and/or *Fgf8* by morpholino injection blocks the effects of exogenous RA (Phillips et al., 2001).

Here, we show that the opposite otic phenotypes generated by more or less RA signaling are produced by different mechanisms. We used a 50-fold lower dose of RA than that of previous studies, and show that RA affects otic induction without expanding *fgf3* and *fgf8* expression in the hindbrain. Instead, this low dose of excess RA leads to an increase in otic competence due to expanded expression of *foxi1* throughout the preplacodal domain. By contrast, reduced RA signaling results in lower induction and impaired maintenance of otic fates, due to compromised *fgf3* and *wnt8b* expression in the hindbrain. We further show that *fgf8* is the primary inducing factor in RA-deprived embryos and that the induction but not the maintenance phenotype can be rescued by an increase in Fgf-signaling.

MATERIALS AND METHODS

Animals

Embryos were obtained from the University of Oregon zebrafish facility, produced using standard procedures (Westerfield, 2000) and staged according to standard criteria (Kimmel et al., 1995) or by hours post

fertilization at 28°C (hpf). The wild-type line used was AB. The lines *acerebellar*^{hi282a} (a strong hypomorphic allele of *fgf8*); *lia*^{t24149} (a hypomorphic allele of *fgf3*); *tcf2*^{hi2169} (a null or strong hypomorphic allele of *tcf2*) and the transgenic line *Tg(hsp:fgf8)* (to misexpress *fgf8*) have been described previously (Brand et al., 1996; Herzog et al., 2004; Sun and Hopkins, 2001; Hans et al., 2007), and we refer to the homozygous mutants as *fgf8*, *fgf3* and *tcf2* mutants, respectively. Homozygous mutants and double mutants were scored either by their morphological phenotype and/or by PCR or loss of *pou1f1* expression (Herzog et al., 2004; Sun and Hopkins, 2001). Heat-shock-treatment embryos were transferred, still in their chorions, into fresh 37-39°C embryo medium in a 1.5 ml tube (20 embryos per tube) and maintained for 30 minutes in a heating block.

Genes and markers

Approved gene and protein names that follow the zebrafish nomenclature conventions (http://zfin.org/zf_info/nomen.html) are used.

In situ hybridization

cDNA probes that detect the following genes were used: *clnna* (Kollmar et al., 2001); *dlx3b* (Eckert et al., 1992); *fgf3* (Phillips et al., 2001); *fgf8* (Reifers et al., 1998); *foxi1* (Solomon et al., 2003); *otx2* (Li et al., 1994); *pax8* (Pfeffer et al., 1998); *pou1f1* (Nica et al., 2004); *stm* (Söllner et al., 2003); and *wnt8b* (Kelly et al., 1995). Probe synthesis and single- or double-color in situ hybridization was performed essentially as previously described (Thisse et al., 1993; Jowett and Yan, 1996; Whitlock and Westerfield, 2000).

Morpholinos (MOs) and pharmacological treatments

The *dlx3b*-MO, *foxi1*-MO and *wnt8b*-MO have been previously described (Liu et al., 2003; Solomon and Fritz, 2002; Kim et al., 2002). For pharmacological treatments, the following stock solutions were made and stored at -80°C: 100 mM 4-(Diethylamino)-benzaldehyde (DEAB; Sigma) in DMSO and 1 mM all-trans retinoic acid (RA; Sigma) in DMSO. For embryo treatments, dilutions of these chemicals were made in embryo medium as follows: DEAB, 10 μ M; and RA, 10, 20 and 1000 nM. Prior to gastrulation (30% epiboly or 4.7 hpf) embryos were removed from their chorions and transferred into Petri dishes containing the treatment solution. Treatments with a teratogenic dose of RA were carried out as described previously (Kudoh et al., 2002); dechorionated embryos at 40% epiboly stage were treated with 1 μ M RA for 80 minutes followed by thorough rinses with embryo medium. For control treatments, sibling embryos were incubated in corresponding dilutions of DMSO. All incubations were conducted in the dark.

RESULTS

Excess or loss of RA signaling interferes with otic induction

It has been shown previously that excess or deficient RA signaling leads to opposite otic phenotypes, but the underlying mechanisms have remained obscure (Perz-Edwards et al., 2001; Phillips et al., 2001). To study the role of RA signaling in otic development, we analyzed the expression of several otic markers at the vesicle, placodal and preplacodal stages. To interfere with RA signaling, we used the pharmacological inhibitor 4-(Diethylamino)-benzaldehyde (DEAB), a potent retinaldehyde dehydrogenase inhibitor (Russo et al., 1988). Compared with the morphology of controls at 50 hpf, loss of RA signaling led to strongly reduced otic vesicles with only one small otolith and impaired semicircular canal protrusions, whereas excess RA at a concentration of 10 nM led to the formation of enlarged otic vesicles containing three otoliths and multiple semicircular canal protrusions (Fig. 1A-C). Assessed at 22 hpf by expression of the otic marker *starmaker* (*stm*), which was expressed throughout the entire epithelium of control otic vesicles, otic vesicle size was reduced by up to 50% in RA-signaling-depleted embryos, but increased up to 40% in the presence of 10 nM RA (Fig. 1D-F). At the 12-somite stage, when the placode was outlined by *clnna* expression in control embryos, the otic placode was reduced in size

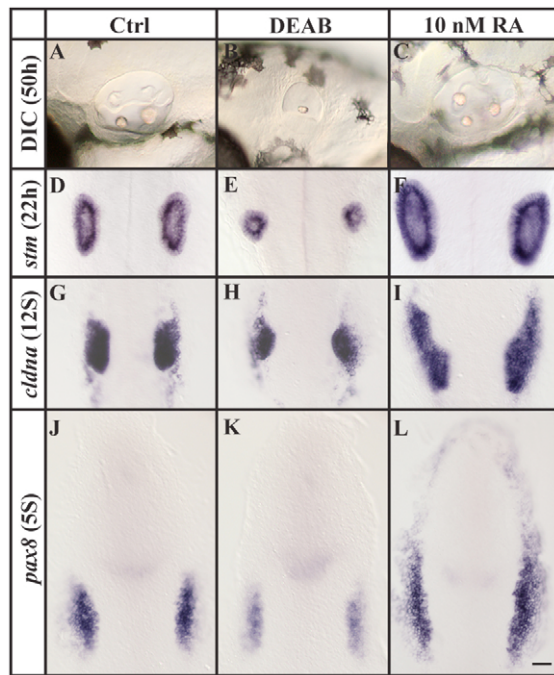


Fig. 1. Loss and excess of RA signaling generate opposite phenotypes. (A-F) Compared to control embryos, otic vesicles are reduced or increased in size, respectively, after depletion of RA signaling (DEAB) or after the application of 10 nM RA, as assessed both by morphology at 50 hpf (A-C) or *stm* expression at 22 hpf (D-F). (G-L) Reduction or increase in the number of preotic cells is already evident at placodal (G-I, 12-somite stage) and preplacodal (J-L, 5-somite stage) stages after labeling with *cldna* (G-I) or *pax8* (J-L). (A-C) Lateral views of live otic vesicles with anterior to the left and dorsal towards the top. (D-L) Dorsal views with anterior towards the top. Scale bar: 35 μ m.

in RA-signaling-depleted embryos, whereas excess RA led to a size increase (Fig. 1G-I). Use of *pax8* expression, which is initiated in the otic anlagen prior to the formation of the placode, showed that the opposite phenotypes generated by excess or deficient RA signaling are already evident at preotic stages. In RA-signaling-depleted embryos, the preotic expression of *pax8* is reduced, whereas, in RA-treated embryos, the preotic *pax8* expression domain is enlarged and weak expression of *pax8* surrounding the entire anterior neural plate can be detected (Fig. 1J-L). An increase of RA to 20 nM amplifies the *pax8* expression surrounding the anterior neural plate (Fig. 2A), consistent with results published previously (Phillips et al., 2001). However, in contrast to Phillips et al., who reported that 20–30% of embryos treated with teratogenic doses (1 μ M) of RA produce ectopic otic vesicles at the anterior limit of the head, we observed only randomly distributed small patches of ectopic otic cells in embryos treated with 20 nM RA (Fig. 2B-D). To demonstrate that our RA treatment did not posteriorize the anterior neural plate in the same manner as typical teratogenic doses of RA (Phillips et al., 2001; Kudoh et al., 2002), we treated embryos with either 20 nM RA or a teratogenic dose of RA and compared them to controls (see Fig. S1 in the supplementary material). Subsequent marker analysis of the anteroposterior axis showed that teratogenic doses, but not 20 nM, of RA significantly altered the expression of early anteroposterior-axis-specific genes. In comparison to controls, expressions of *six3a* and *otx2* was unaffected by 20 nM RA but are completely abolished in the

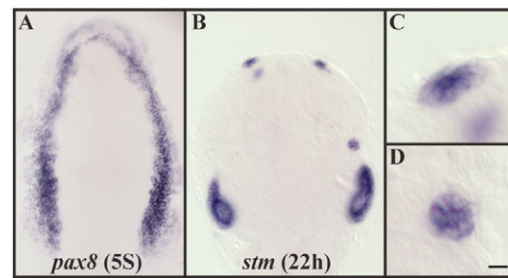


Fig. 2. Application of 20 nM RA leads to widespread ectopic otic induction and to the formation of ectopic otic cells outside of the endogenous ear. (A) Application of 20 nM RA leads to ectopic *pax8* expression encompassing the entire preplacodal domain (compare with Fig. 1J,L). (B-D) Embryos treated with 20 nM RA display ectopic patches of cells that express the otic marker *stm* and form enlarged vesicles. (C,D) Higher magnifications of B show the epithelial structure of these patches. Dorsal views with anterior towards the top. Scale bar: 35 μ m for A; 50 μ m for B; 10 μ m for C,D.

presence of a teratogenic dose (see Fig. S1A-F in the supplementary material). Expression of the RA-controlled gene *hoxb1b* (Alexandre et al., 1996) in the caudal hindbrain of control embryos was mildly expanded in embryos treated with 20 nM RA, but it was strongly misexpressed throughout the entire neural plate of embryos treated with a teratogenic dose of RA (see Fig. S1G-I in the supplementary material). In embryos treated with 20 nM RA, expression of *gbx1* in the rostral hindbrain was indistinguishable from control embryos, whereas it was misexpressed in the anterior neural plate of embryos treated with a teratogenic dose of RA (see Fig. S1J-L in the supplementary material). Together, our results show that the opposite phenotypes generated by gain or loss of RA signaling can be traced back to otic induction and that ectopic otic induction occurs with low doses of RA that do not apparently affect other aspects of embryonic development.

Excess RA induces *foxi1*, whereas loss of RA signaling reduces *fgf3* expression

Our results indicate that loss of RA signaling impairs the formation of the otic vesicle, whereas increased RA results in enhanced otic development. This could be due to changes in the mechanisms underlying the induction of otic fates or in the competence of cells to respond to the inductive signals, or both. To distinguish among these possibilities, we examined the expression of the factors that regulate otic induction and competence. In zebrafish, *Fgf3* and *Fgf8* have been implicated to have overlapping functions in otic placode induction (Phillips et al., 2001; Maroon et al., 2002; Léger and Brand, 2002). At 95% epiboly, *fgf3* was expressed strongly in the r4 primordium of control embryos, but its expression was severely reduced in embryos depleted of RA signaling (Fig. 3A,B). Because the complete loss of RA signaling led to a loss of r5–r7 accompanied by an expansion of r3–r4, we consistently observed enlarged *fgf3* and *fgf8* expression domains in DEAB-treated embryos at the three-somite stage, although the level of *fgf3* expression appeared to still be reduced in comparison with control embryos (Fig. 3D,E,G,H). By contrast, expression of both *fgf3* and *fgf8* was completely unaffected in embryos treated with 10 or 20 nM RA (Fig. 3C,F,I and data not shown). We and others recently proposed that *Foxi1* and *Dlx3b* provide competence for cells to form the inner ear (Hans et al., 2004; Solomon et al., 2004). In comparison to control embryos, *foxi1* expression was unchanged in the absence of RA signaling, whereas in embryos treated with 20 nM RA, *foxi1* was

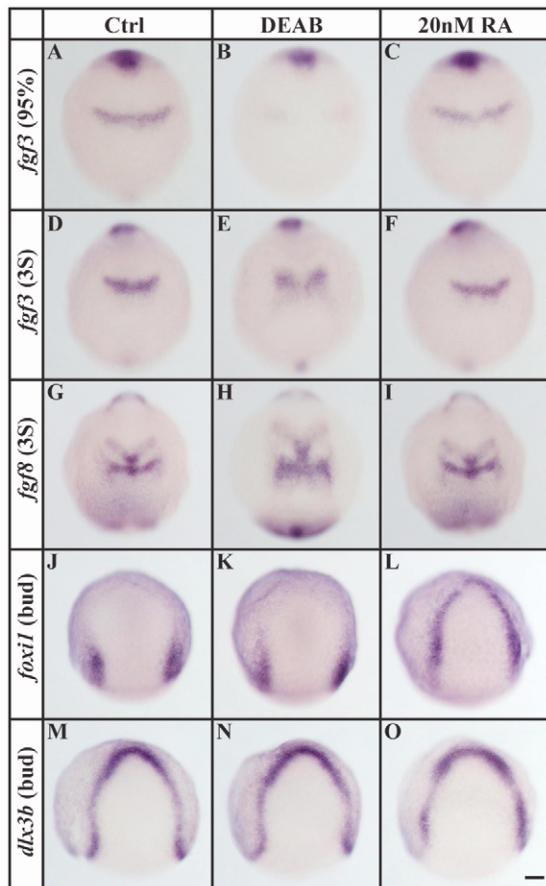


Fig. 3. Loss and excess of RA signaling affect different tissues.

(A-F) In comparison to controls (A,D) or to embryos treated with 20 nM RA (C,F), r4-specific *fgf3* expression is delayed by the end of gastrulation in embryos depleted of RA signaling (DEAB, B) and remains reduced in an enlarged r4 primordium at the three-somite stage (E). (G-I) An enlarged r4-specific *fgf8* expression domain is also present in embryos depleted of RA signaling (H) compared with control (G) or 20 nM RA-treated (I) embryos. (J-L) *foxi1* expression is indistinguishable from control embryos (J) after RA-signaling depletion (K), whereas the application of 20 nM RA leads to ectopic expression within the entire preplacodal domain (L). (M-O) Expression of *dlx3b* in the preplacodal domain is unaffected by loss (N) or excess (O) of RA signaling. Dorsal views with anterior towards the top. Scale bar: 90 μ m.

misexpressed in a stripe surrounding the anterior neural plate (Fig. 3J-L). By contrast, we observed no change in *dlx3b* expression after the complete loss or ectopic activation of RA signaling (Fig. 3M-O). These results show that the complete loss of RA signaling impairs early *fgf3* expression in the developing hindbrain, which cannot be compensated for by expanded hindbrain-specific domains of *fgf3* and *fgf8* expression at later stages. Excess RA signaling, on the other hand, causes an expansion of *foxi1* expression throughout the entire preplacodal ectoderm.

Lens development is disturbed in the presence of ectopic RA signaling

Our observation that *foxi1* is misexpressed in RA-treated embryos suggested the possibility that the preplacodal domain is posteriorized in these embryos, similar to fate changes in the developing neural plate in embryos treated with a teratogenic dose of RA (Marshall et al., 1992; Kudoh et al., 2002). Subsequently, other placode-derived

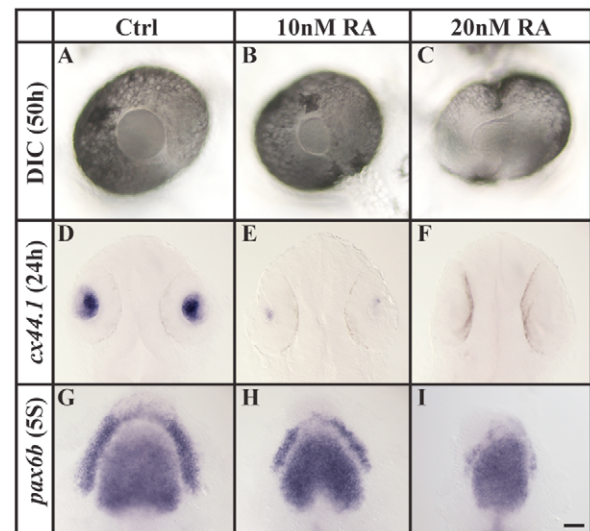


Fig. 4. Increased RA signaling compromises lens specification.

(A-F) Assessed both by morphology at 50 hpf (DIC microscopy, A-C) and *cx44.1* expression at 24 hpf (D-F), the lens is reduced in size or completely lost after the application of 10 or 20 nM RA, compared with wild-type embryos. (G-I) Compromised lens specification is already evident at preplacodal stages (five-somite stage, 5S), as indicated by *pax6b* labeling. (A-C) Lateral views of live eyes with anterior to the left and dorsal towards the top. (D-I) Dorsal views with anterior towards the top. Scale bar: 30 μ m for A-C; 50 μ m for D-F; 35 μ m for G-I.

structures might be affected. To examine this possibility, we tested molecular markers for the formation of anterior pituitary, olfactory, lens, trigeminal, lateral line and epibranchial placodes. Except for the lens, all of these structures formed in embryos treated with 20 nM RA (data not shown). Compared with controls at 50 hpf, embryos treated with 10 nM RA developed a much smaller lens and embryos treated with 20 nM RA completely failed to show any morphological sign of a lens (Fig. 4A-C). Consistent with this, expression of *connexin 44.1*, which is expressed only in the ocular lens (Cason et al., 2001), was changed. In comparison to controls at 24 hpf, embryos treated with 10 or 20 nM RA showed severe downregulation or no expression of *connexin 44.1*, respectively (Fig. 4D-F). Labeling for *pitx3*, which is expressed in the ventral and posterior diencephalon, in the lens and in the pituitary placode at 24 hpf in wild-type embryos (Dutta et al., 2005), corroborated our results, showing expression changes, after RA treatment, specifically in the lens placode without affecting other expression domains (data not shown). At early segmentation stages, *pitx3* expression defines an equivalence domain for the lens and anterior pituitary placodes (Dutta et al., 2005), and we found that its expression was unaffected in the presence of 10 or 20 nM RA (data not shown). By contrast, expression of *pax6b*, a specific lens placode marker (Thisse et al., 2001), was downregulated or almost absent in embryos treated with 10 or 20 nM RA, respectively, in comparison to control embryos (Fig. 4G-I). Together, our results show that increasing amounts of RA impair lens placode formation, which subsequently leads to a loss of the lens.

Residual otic induction in RA-depleted embryos is primarily due to Fgf8 signaling

In zebrafish, Fgf3 and Fgf8 are necessary for otic induction and, because loss of RA signaling led to a significant reduction in *fgf3* expression in the developing hindbrain, we hypothesized that the

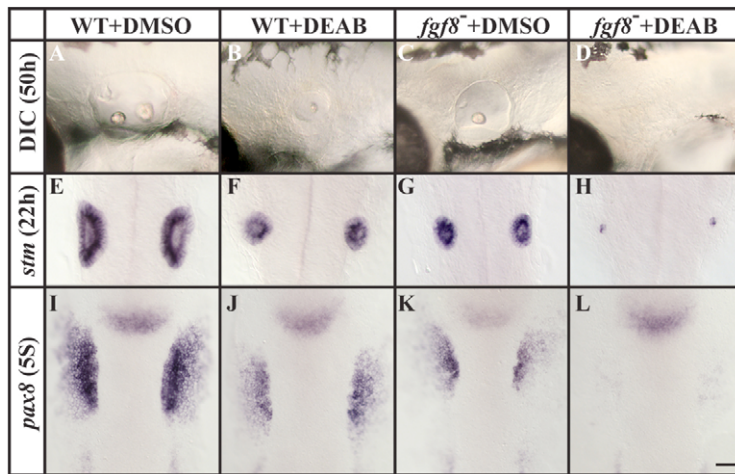


Fig. 5. Fgf8 mediates otic induction in the absence of RA signaling. (A-D) Assessed by morphology at 50 hpf (DIC microscopy), otic vesicles are reduced after the depletion of RA (B) and in *fgf8* mutants (C), and are completely lost in *fgf8* mutants depleted of RA signaling (D), in comparison to controls (A). (E-H) Labeling with *stm* at 22 hpf reveals the presence of only residual otic cells in *fgf8* mutants depleted of RA signaling (H) compared to *fgf8* mutant (G), RA-depleted (F) or control (E) embryos, in which more otic cells are present. (I-L) Otic vesicle size reduction in *fgf8* mutants depleted of RA signaling is evident as early as the preplacodal stages (five-somite stage, 5S), as detected by labeling with *pax8*. (A-D) Lateral views of live otic vesicles with anterior to the left and dorsal towards the top. (D-L) Dorsal views with anterior towards the top. Scale bar: 35 μ m.

remaining otic induction is dependent primarily on *fgf8*. In comparison to controls, otic vesicles in the absence of RA signaling or in *fgf8* mutants are reduced in size and typically form only one otolith (Fig. 5A-C). By contrast, loss of RA signaling in *fgf8* mutants produced a different and highly penetrant phenotype ($n=25$ out of 25 *fgf8* mutants); embryos never formed an otic vesicle (Fig. 5D). Nevertheless, the otic marker *stm* revealed the presence of some residual otic cells in *fgf8* mutants ($n=10$ out of 10 *fgf8* mutants) depleted of RA signaling compared to untreated *fgf8* mutants, RA-depleted or control embryos (Fig. 5E-H). Analysis of *pax8* expression showed that this phenotype was already evident at preotic stages. Loss of *fgf8* or RA signaling alone led to reduced *pax8* expression in the preotic region, but combined loss of *fgf8* and RA signaling caused a severe loss of *pax8* (Fig. 5I-L). Our observation of some indications of residual otic specification in *fgf8* mutants depleted of RA signaling is consistent with the delayed and reduced expression of *fgf3* in the hindbrain. Furthermore, loss of RA signaling in *fgf3* mutants had no effect on otic vesicle size (data not shown), supporting our hypothesis that, in the absence of RA signaling, residual otic induction depends primarily on Fgf8.

Otic induction can be rescued but is not maintained in RA-depleted embryos

Our results indicate that competence provided by Foxi1 and Dlx3b to initiate otic fate is not impaired in RA-depleted embryos. This suggests that reduced otic induction caused by the delayed onset of *fgf3* expression is responsible for the observed otic phenotype, which we hypothesize could be rescued by ectopic Fgf signaling. To activate ectopic Fgf signaling, we used a stable transgenic line that allows us to express *fgf8* uniformly under the control of the zebrafish temperature-inducible *hsp70* promoter (Hans et al., 2007). Misexpression of *fgf8* at late gastrulation stages led to ectopic otic induction and caused expanded marker expression, such as that of *pax8*, *pax2a* and *sox9a*, within the preotic region in comparison to non-transgenic siblings (Fig. 6A,B) (Hans et al., 2007). Subsequently, many more cells embarked on the otic development pathway and much larger otic vesicles formed (Fig. 6E,F) (Hans et al., 2007). By contrast, we also observed an expanded *pax8* expression domain after misexpression of *fgf8* at late gastrulation stages in embryos depleted of RA signaling, but the subsequent formation of enlarged otic vesicles was impaired (Fig. 6C,D,G,H). In embryos depleted of RA signaling, otic vesicles were only slightly

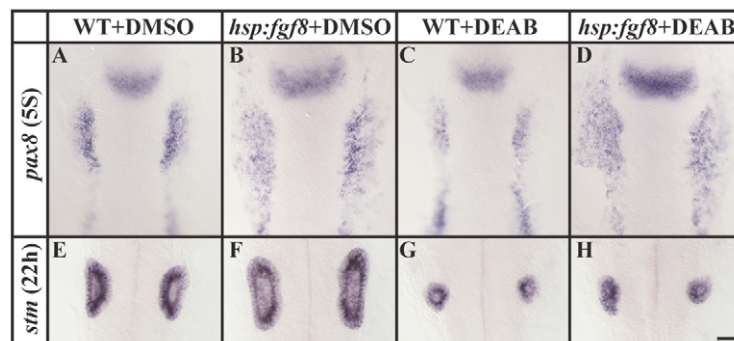


Fig. 6. Misexpression of *fgf8* at the end of gastrulation rescues otic induction in RA-depleted embryos, but the rescue is not maintained. (A,B) Preotic *pax8* expression is expanded in transgenic *hsp:fgf8* embryos (B) after misexpression of *fgf8* at the end of gastrulation in comparison to non-transgenic embryos (A). (C,D) Reduced preotic *pax8* expression in RA-depleted embryos (C) can be rescued by the misexpression of *fgf8* at the end of gastrulation (D), as can heat-shocked transgenic *hsp:fgf8* embryos with normal RA signaling (B). (E,F) Misexpression of *fgf8* at late gastrulation stages (F) leads to the formation of much larger otic vesicles in comparison to their non-transgenic siblings (E). (G,H) Transgenic *hsp:fgf8* embryos depleted of RA signaling (H) do not show an increase in otic cells but rather resemble non-transgenic RA-depleted siblings (G). Dorsal views with anterior towards the top. Scale bar: 35 μ m.

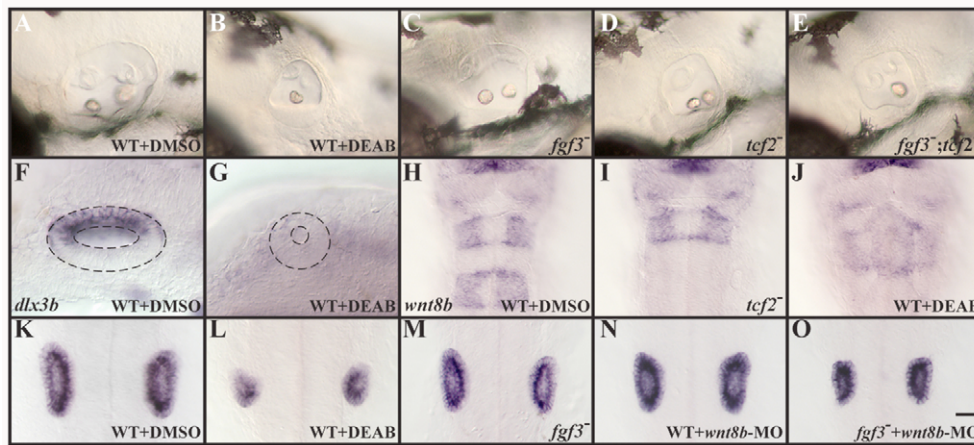


Fig. 7. Rhombomere 5-specific Wnt8b function is required for the maintenance of otic fate. (A–D) Assessed by morphology, otic vesicles are reduced in *fgf3* (C) and *tcf2* (D) single mutants compared to wild-type embryos (A), but not as much as in RA-depleted embryos (B). (E) Loss of both *fgf3* and *tcf2* together results in otic vesicles that form only one otolith, as is observed in RA-depleted embryos (B). (F,G) Expression of *dlx3b* in the dorsal half of the otocyst at 22 hpf is lost in embryos depleted of RA signaling. (H–J) In control embryos at 22 hpf, *wnt8b* expression can be detected in the midbrain-hindbrain boundary, in r3 and in r5 (H), whereas *tcf2* mutants (I) and RA-depleted embryos (J) show *wnt8b* in the midbrain-hindbrain boundary and in r3, but not in r5. (K–N) Inactivation of Wnt8b in wild-type embryos by morpholino injection (MO, N) leads to a reduced ear size (labeled with *stm* at 22 hpf), similar to the size observed in *fgf3* mutants (M), compared to untreated wild type (K); however, the size reduction is not as severe as in embryos depleted of RA signaling (L). Loss of Wnt8b function in *fgf3* mutants (O) produces reduced otic vesicles comparable to RA-depleted embryos (L). (A–G) Lateral views with anterior to the left and dorsal towards the top. (H–O) Dorsal views with anterior towards the top. Scale bar: 35 μ m for A–E and H–O; 20 μ m for F,G.

bigger after ectopic activation of Fgf signaling at late gastrulation stages than in non-transgenic siblings depleted of RA signaling, and they were still much smaller than in untreated controls. We also frequently observed small ectopic otic vesicles located anteriorly and posteriorly to the endogenous otic vesicle after misexpression of *fgf8* at late gastrulation stages in RA-depleted embryos (data not shown), but, in general, otic specification was always reduced in comparison to control embryos. Taken together, our results demonstrate that, in the absence of RA signaling, cells in the preotic region can undergo ectopic otic induction, similar to control embryos, after the ectopic activation of Fgf signaling at late gastrulation stages. However, otic fate cannot be properly maintained in the absence of RA signaling.

Wnt8b is required to maintain otic fate

Our results suggested that an Fgf-independent mechanism is required to maintain otic fate and, because loss of RA signaling led to the expansion of r3–r4 accompanied by loss of r5–r7, we hypothesized that the source might be located within r5–r7. Analysis of *fgf3*;*tcf2* double mutants confirmed this hypothesis (Fig. 7A–E). The homeobox gene *tcf2* (previously *vhnl1*) is expressed in the posterior hindbrain and anterior spinal cord in an RA-dependent manner at early segmentation stages. The anterior borders of the *tcf2* expression domain and of r5 coincide (Sun and Hopkins, 2001; Hernandez et al., 2004). In strong *tcf2* mutant alleles, the r5–r6 region of the developing hindbrain is lost and partially transformed into an r4 identity (Hernandez et al., 2004). Although otic induction is unaffected at early stages in these mutants (Sun and Hopkins, 2001), otic vesicles are reduced in size at later stages indicating that maintenance of otic fate is compromised (Fig. 7A,D) (Sun and Hopkins, 2001). In comparison to either wild type or *tcf2* or *fgf3* single mutants, combined loss of *tcf2* and *fgf3* resulted in otic vesicles that formed only one otolith ($n=10$ out of 10 *fgf3*;*tcf2* double mutants), similar to RA-depleted embryos (Fig. 7A–E). Semicircular canal formation, however, which was severely

impaired in RA-signaling-depleted embryos, was less severely affected in *fgf3*;*tcf2* double mutants, indicating that, similar to mouse (Romand, 2003), RA signaling is involved in pattern formation of the zebrafish otic vesicle. Recent studies in mouse have shown that Wnt signaling is required early for the maintenance of otic fate and later in the otic vesicle for expression of *dlx5* and for the acquisition of a dorsal fate (Ohya et al., 2006; Riccomagno et al., 2005). We found that expression of *dlx3b* in the dorsal portion of the otic vesicle was completely lost in RA-depleted embryos at 22 hpf (Fig. 7F,G), suggesting that Wnt signaling may be affected. Wnt8b is a likely candidate because it is expressed strongly in r1, r3 and r5 from mid-somitogenesis onwards (Kelly et al., 1995). Compared to controls, both *tcf2* mutants and RA-depleted embryos showed a loss of *wnt8b* expression specifically in r5 (Fig. 7H–J). To test whether *wnt8b* acts to maintain otic fate, we compromised Wnt8b function using morpholino injection into wild type. Furthermore, we compromised Wnt8b function in *fgf3* mutants to test whether loss of *fgf3* and *wnt8b* together mimics the otic vesicle size reduction of RA-signaling-depleted embryos, which have defects in *fgf3* and *wnt8b* expression (Fig. 7K–O). Loss of Fgf3 or Wnt8b function alone led to the formation of smaller otic vesicles, but the size reduction was not as profound as in embryos depleted of RA signaling (Fig. 7K–N). However, compromised Wnt8b function in *fgf3* mutants showed an additive effect, producing smaller otic vesicles similar to vesicles in RA-depleted embryos ($n=14$ out of 18) (Fig. 7L,O). Depletion of RA signaling and Wnt8b function together resulted in a slight further reduction in otic vesicle size (data not shown). However, because of the expansion of r3–r4, otic vesicles were located closer to r3 in RA-depleted embryos (data not shown), and expression of *wnt8b* in r3 might partly compensate for the loss of *wnt8b* from r5 in these embryos. Together, our results demonstrate that the hindbrain-derived factor Wnt8b from r5 maintains otic fate, and that compromised Fgf3 expression and loss of Wnt8b function together mimics the size reduction of the otic vesicle observed in RA-depleted embryos.

DISCUSSION

Ectopic RA signaling increases the competence of cells to respond to otic-inducing signals, but endogenous RA is not required for otic competence

Treatment of vertebrate embryos with RA causes a posteriorization of the anterior neural plate in a concentration-dependent manner, which leads to transformation of r2-r3 into r4-r5 and to the expansion of the posterior hindbrain at the expense of the presumptive fore- and mid-brain structures (Marshall et al., 1992; Kudoh et al., 2002). Furthermore, teratogenic doses of RA completely transform forebrain and midbrain into hindbrain and enlarge the preotic expression domain of *pax8* (Phillips et al., 2001). Our results demonstrate that much lower concentrations of RA that do not interfere with the patterning of the anterior neural plate are sufficient to produce ectopic expression of *foxi1*, which expands the domain of preotic *pax8* expression. Consistent with recent reports showing that preotic *pax8* expression depends on Foxi1 (Solomon et al., 2003; Nissen et al., 2003), we found that the enlarged *pax8* expression observed in 20 nM RA-treated wild-type embryos was severely reduced to a small, residual domain of expression at the anterior end in RA-treated *foxi1* mutants (Hans et al., 2007). Fate changes after the application of 20 nM RA were observed only in the preplacodal domain, but not within the anterior neural plate, whereas a teratogenic dose of RA affected both tissues. This difference is presumably due to the presence of RA-degrading enzymes of the Cyp26 class that are expressed within the anterior neural plate but not in the preplacodal domain (Dobbs-McAuliffe et al., 2004; Kudoh et al., 2002; Hernandez et al., 2006). Consistent with this interpretation, a recent study has shown that 5 nM RA is sufficient to posteriorize cyp26a1 mutants, whereas 200 nM RA is required to obtain the same effect on wild-type embryos (Hernandez et al., 2006).

Our observation that excess RA leads to ectopic expression of *foxi1* throughout the entire preplacodal domain provides a consistent explanation for the findings of Woo and Fraser (Woo and Fraser, 1997). Transplantation of ventral and lateral germring tissue into the prospective forebrain region can induce ectopic otic vesicles, whereas the transplantation of dorsal germring tissue (the embryonic shield) does not. Although germring grafts also lead to the induction of ectopic hindbrain tissue, the direct transplantation of hindbrain into the prospective forebrain region never induces ectopic otic vesicles (Woo and Fraser, 1997). RA is generated by the enzymatic activity of aldehyde dehydrogenases, which are encoded by *Aldh* genes, and, during zebrafish gastrulation, *aldh1a2* is expressed in the ventral and lateral germring but not dorsally in the embryonic shield (Begemann et al., 2001; Grandel et al., 2002). Thus, we propose that RA produced by the grafted ventral and lateral germring increases the competence of anterior preplacodal cells to respond to otic-inducing signals because of ectopic activation of *foxi1*. The observed ectopic otic induction might also benefit from ectopic Fgf signaling provided by the graft, because *fgf3*, *fgf8*, *fgf17b* and *fgf24* are all expressed in a dorsoventral gradient in the zebrafish germ ring, with highest levels on the dorsal side (Fürthauer et al., 1997; Fürthauer et al., 2004; Reifers et al., 1998; Cao et al., 2004).

Although RA is sufficient to induce *foxi1* expression, our data also show that loss of RA signaling during zebrafish gastrulation has no effect on the expression of *foxi1*. Thus, impaired otic induction in RA-depleted embryos is not due to an effect on Foxi1 level and, hence, we conclude that RA signaling is not required for the early events of otic induction. The observed effect of excess RA on *foxi1* expression might rather reveal a regulatory mechanism employed

during other aspects of development. In zebrafish at 48 hpf, *foxi1* is expressed in the retina, in the pharyngeal pouches and in the dorsal septum of the otic vesicle (Thisse et al., 2005), and RA signaling has been implicated in the development of all of these tissues (Dräger et al., 2001; Mark et al., 2004; Romand, 2003). It is currently unknown, however, whether *foxi1* expression depends on RA signaling in these tissues.

Our results further indicate that early *fgf3* expression is crucial during the relatively short time-window for otic induction in zebrafish. Impaired *fgf3* expression in the developing hindbrain of RA-signaling-depleted embryos delayed otic induction and could not be compensated for by an expanded hindbrain domain of *fgf3* and *fgf8* expression at later stages. Consistently, depletion of *fgf3* by morpholino injection severely reduces *pax8* expression at the tailbud stage (Phillips et al., 2001; Léger and Brand, 2002). Based on experiments with a stable transgenic line that expresses *fgf8* uniformly under the control of the zebrafish temperature-inducible *hsp70* promoter, we recently proposed that otic induction in zebrafish occurs between the end of gastrulation and the early segmentation stages (Hans et al., 2007). Using the same approach here, we show that even in the absence of RA signaling, cells in the preotic region can undergo ectopic otic induction after ectopic activation of Fgf signaling at late gastrulation stages, similar to control embryos. Thus, cells are still competent to adopt otic fate after exposure to a high uniform dose of Fgf8 but are unable to respond to the endogenous Fgf3 and Fgf8 emanating from the developing hindbrain after the loss of RA signaling. Incorrect spatial positioning of inducing and induced tissue might also contribute to impaired otic induction, because, in RA-signaling-depleted embryos, the r3 primordium is greatly expanded, displacing the inducing r4 from the preotic region.

Wnt signaling during otic induction

Our analysis further demonstrates that defects in otic specification caused by loss of RA signaling are a secondary consequence of changes in hindbrain patterning. We found that embryos depleted of RA signaling formed small otic vesicles because of compromised otic induction due to decreased *fgf3* expression and because of compromised maintenance of otic fate due to loss of *wnt8b* expression. Reduced otic induction due to weaker expression of Fgf3 has been proposed from work in both mammals and zebrafish (Niederreither et al., 1999; Whitfield et al., 2002; Phillips et al., 2001; Léger and Brand, 2002), and a recent study in mouse shows that Wnt signaling is required after Fgf-dependent induction to maintain otic fate (Ohyama et al., 2006). In the latter study, Wnt signaling was detected using a Tcf/Lef reporter construct within the preotic region in a subset of Pax2-positive cells after Fgf-dependent induction but prior to the formation of the placode. Furthermore, disruption of the canonical Wnt signaling pathway in preotic Pax2-positive cells leads to an expansion of epidermal fate at the expense of otic fate, and otic vesicles are significantly reduced in size (Ohyama et al., 2006). Conversely, constitutive activation of canonical Wnt signaling in preotic Pax2-positive cells leads to the expansion of otic fate at the expense of epidermal fate, suggesting that Wnt signaling mediates a placode-epidermis fate decision by directing preotic cells to an otic fate (Ohyama et al., 2006). So far, it is unknown whether Wnt signaling plays the same role in zebrafish. It has been reported that, in zebrafish, the Wnt reporter gene *TOPdGFP*, a transgene consisting of a GFP-coding sequence downstream of a minimal promoter and four Lef-binding sites, is not expressed during early stages of otic induction (Phillips et al., 2004), but nothing is known about its expression later in development, prior

to the formation of the otic placode. Consistent with a similar role for Wnt signaling, we found that, in RA-depleted embryos, otic induction can be rescued but not maintained. Furthermore, we have previously found that expression of the endogenous Wnt reporter gene *axin2* is absent during the initial stages of otic induction but that *axin2* is expressed within the preotic region prior to the formation of the otic region (our unpublished results). Cell-autonomous disruption or constitutive activation of the canonical Wnt signaling pathway in mouse by conditional knockout of β -catenin or by the expression of a conditionally activated form of β -catenin within preotic Pax2-positive cells led Ohyama et al. to propose that *Wnt8a* from r4 is the source of Wnt signaling (Ohyama et al., 2006). Our data, on the other hand, indicate that *wnt8b* expression in r5 is the source, consistent with findings that Wnt8b signaling is strongly deficient in the posterior hindbrain of *maf8* mutants and that inactivation of Wnt8b by morpholino injection results in smaller otic vesicles that mimic the size and patterning defects of *maf8* mutants (M. Brand, personal communication). In zebrafish, *wnt8a* encodes a bicistronic message encoding two complete open-reading frames, ORF1 and ORF2, and, at 75% epiboly (8 hpf), ORF2 can be detected in r5-r6 adjacent to the otic anlagen (Kelly et al., 1995; Lekven et al., 2001). Loss of Wnt8a function by morpholino injection results in greatly reduced otocysts (Phillips et al., 2004), supporting the model proposed by Ohyama et al. (Ohyama et al., 2006). However, proper expression of *fgf3* and *fgf8* is disturbed in the hindbrain of these embryos, thus compromising otic induction (Phillips et al., 2004) and making it difficult to address later Wnt8a function during otic development. It is very likely that several Wnt molecules are involved in otic fate maintenance after Fgf-dependent induction. Expression of *wnt1*, *wnt3a* and *wnt10b* has been reported in the zebrafish hindbrain in addition to *wnt8a* and *wnt8b*, and, interestingly, expression of *wnt1* and *wnt3a* is severely reduced in the caudal hindbrain of *tcf2* mutants (Lekven et al., 2003; Buckles et al., 2004; Lecaudey et al., 2006), as we have shown for *wnt8b*. Conditional and combinatorial knockout will be necessary to address the role of particular Wnt molecules in otic fate maintenance.

Absent or inappropriate amounts of Wnt signaling might also be responsible for the reduced number of otic cells in RA-treated embryos at later stages. Our treatment of embryos with 20 nM RA led to the massive ectopic expression of *pax8* around the anterior neural plate border, similar to the results published by Phillips et al. (Phillips et al., 2001). However, in contrast to Phillips et al., who reported that 20–30% of RA-treated embryos produce ectopic morphologically visible otic vesicles at the anterior limit of the head, we observed only randomly distributed small patches of ectopic otic cells. Phillips et al. showed that the anterior neural plate is strongly posteriorized after treatment with a teratogenic dose of RA, which leads to the anterior expansion of hindbrain *fgf3* and *fgf8* expression domains. Wnt signaling is presumably also shifted anteriorly in these RA-treated embryos, maintaining the fate of the ectopic otic cells. In wild-type embryos, however, Wnt signaling is absent in the early neural plate anterior to the future midbrain-hindbrain boundary (Wilson and Houart, 2004). Because our conditions to generate excess RA signaling (20 nM) did not apparently change anterior neural plate fate, *pax8*-positive cells anterior to the future midbrain-hindbrain boundary presumably do not receive Wnt signaling, which is essential for the maintenance of otic fates after induction. Furthermore, pathways to generate other placode-derived sensory organs were presumably intact under our experiments, which might also contribute to fewer ectopic otic cells in RA-treated embryos at later stages.

Ectopic RA signaling and lens placode induction

We found that the application of 20 nM RA led to the expression of *foxi1* within the entire preplacodal domain. However, we did not observe any expansion of the otic tissue at the expense of other sensory organs, except for the lens. Interestingly, it was recently shown that the entire preplacodal domain is initially specified as lens tissue, implying that lens fate is a default state of the preplacodal territory and that subsequent development requires repression of lens fate in prospective non-lens domains (Bailey et al., 2006). By promoting otic fate in response to Fgf signaling, Foxi1 in the preotic region of wild-type embryos and expanded Foxi1 expression in RA-treated embryos might be involved in lens fate repression. Consistent with this interpretation, microarray analysis covering approximately 20,000 zebrafish genes showed that only seven genes, including *pax6b*, are significantly repressed after overexpression of Foxi1 (Yan et al., 2006). However, it is also likely that the reduction or loss of lens tissue is not due to misexpression of *foxi1* or to a posteriorization of the preplacodal domain, but rather due to the ectopic expression of *pax8*. Reciprocal inhibition between the paired-domain proteins Pax2 and Pax6 has been implicated in the specification of mammalian eye territories and in formation of the di-mesencephalic boundary (Schwarz et al., 2000; Nakamura and Watanabe, 2005). Other studies have shown that both Pax2 and Pax6 can be converted from transcriptional activators to repressors via interaction with co-repressors of the Groucho protein family (Eberhard et al., 2000). Pax2 is highly related to Pax5 and Pax8, and gene replacement in the mouse has shown that Pax5 can functionally substitute for Pax2, indicating that Pax2, Pax5 and Pax8 proteins are biochemically interchangeable (Pfeffer et al., 1998; Bouchard et al., 2000). Combined and redundant gene function has also been shown for Pax2 and Pax8 during development of the mouse urogenital system and the zebrafish inner ear (Bouchard et al., 2002; Hans et al., 2004; Mackereth et al., 2005). Further investigation of the roles of Foxi1 and Pax2 or Pax8 in otic induction and in the repression of other fates will help clarify the functional similarities and differences among these factors.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/13/2449/DC1>

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