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Retinoid Signaling in Inner Ear Development: a “Goldilocks” Phenomenon

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Abstract

Retinoic acid is a biologically active derivative of vitamin A that is indispensable for inner ear development. The normal function of retinoic acid is achieved only at optimal homeostatic concentrations, with an excess or deficiency in retinoic acid leading to inner ear dysmorphogenesis. We present an overview of the role of retinoic acid in the developing mammalian inner ear, discussing both how and when retinoic acid may act to critically control a program of inner ear development. Molecular mechanisms of otic teratogenicity involving two members of the fibroblast growth factor family, *FGF3* and *FGF10*, and their downstream targets, *Dlx5* and *Dlx6*, are examined under conditions of both retinoic acid excess and deficiency. We term the effect of too little or too much retinoic acid on *FGF/Dlx* signaling a Goldilocks phenomenon. We demonstrate that in each case (retinoic acid excess, retinoic acid deficiency), retinoic acid can directly affect *FGF3/FGF10* signaling within the otic epithelium, leading to downregulated expression of these essential signaling molecules, which in turn, leads to diminution in *Dlx5/Dlx6* expression. Non-cell autonomous effects of the otic epithelium subsequently occur, altering transforming growth factor beta (TGF β) expression in the neighboring periotic mesenchyme and serving as a putative explanation for retinoic acid-mediated otic capsule defects. We conclude that retinoic acid coordinates inner ear morphogenesis by

controlling an *FGF/Dlx* signaling cascade, whose perturbation by deviations in local retinoid concentrations can lead to inner ear dysmorphogenesis.

Keywords

Retinoic acid; inner ear; dysmorphogenesis; fibroblast growth factor; Dlx

INTRODUCTION

Gene-environment interactions are integral to understanding birth defects and their causes. Hearing loss is one of the most common birth defects, with inner ear development being significantly influenced by both nature and nurture. Exposure to teratogenic agents and inadequate nutrition during pregnancy are known environmental factors that can lead to congenital hearing impairment through a variety of methods, including modifying gene expression. Retinoic acid (RA), a biologically active derivative of vitamin A, falls into both these categories, acting as a potent teratogen when exceeding homeostatic concentrations and as an essential morphogen whose deficiency is attributed to otic dysmorphogenesis.

RA, its nuclear receptors, and the enzymes required for RA synthesis are endogenous to the developing inner ear [Biesalski, 1989; Romand et al., 2002, 2006], suggesting a requirement for RA during inner ear development. The critical developmental window during which RA is essential may be compromised if maternal vitamin A intake is marginal [Zile, 2001], leading to inner ear embryopathies and impaired inner ear functionality. In animal models, the developing inner ear is particularly vulnerable to vitamin A deficiency, with even partial deficiency resulting in the formation of abnormally small otic vesicles, absent endolymphatic ducts, atrophy of vestibular and cochlear epithelium, hypertrophy of the otic capsule, degenerative changes in ganglion cells and often a more caudal location [Chole and Quick, 1976; Lohle, 1985; Maden et al., 1996; White et al., 2000]. Absence of the otic vesicle can result from complete elimination of dietary vitamin A [Kil et al., 2005]. Mice carrying a targeted mutation of retinoid receptors or RA metabolizing enzymes that leads to deficiencies in endogenous retinoid signaling exhibit otic anomalies that recapitulate vitamin A deficiency in animal models [Niederreither et al., 1999; Romand et al., 2002]. Thus a wealth of data illustrates loss of RA signaling leads to aberrant otic development.

Exposure to excess retinoic acid is also harmful for otic development. Children with a history of embryonic exposure to retinoids are at a risk for microtia and anotia, typically with low set remnants of pinnae and stenotic external acoustic meati, as well as malformations of the inner ear, the severity of which appears to be correlated with the severity of the external ear malformation [Berger, 2004; Lammer, 1985]. Two basic types of inner ear defects are induced by excess retinoids, i.e. Michel aplasia and the Mondini-Alexander defect. Children with an isotretinoin-induced Mondini defect (a retinoid-based acne cream/cancer medication) are characterized by a cochlea with fewer turns than normal, marked loss of cochlear neurons and an abnormally large utricle and saccule. Extreme cases of embryopathy present with a Michel aplasia in which there is complete arrest of inner ear development at the otocyst stage or formation of a vestigial inner ear with or without sensory innervation [Berger, 2004]. Retinoid mediated inner ear defects affecting the cochlea, semicircular canals, utricle, saccule, cochleovestibular ganglia, and otic capsule (i.e. Mondini-like embryopathies) have been reproduced in animal models, with severe cases typified by formation of a cystic structure without distinct organization into cochlear or vestibular portions (i.e. Michel-like aplasia) [Frenz et al., 1996; Burk and Wilhite, 1992; Granström, 1990; Jarvis et al., 1990].

Thus it is clear that regulation of local retinoid concentrations is essential to appropriate patterning and development of the inner ear. In this article, we provide an overview of the critical stages of inner ear development that may be perturbed by RA and discuss the molecular mechanisms that underlie retinoid mediated inner ear embryopathies. We describe how both retinoid excess and deficiency affect a critical inner ear signaling cascade, fibroblast growth factor (*FGF*) signaling and the effects on some of the FGF downstream target genes *Dlx5* and - *Dlx6*, that may explain in part the molecular mechanisms underlying RA-induced abnormal development. We term this effect a “Goldilocks” phenomenon, and discuss its implications for retinoid mediated developmental control.

MATERIALS AND METHODS

Experimental animals

Time-staged pregnant CD-1 mice were purchased from Charles River (Wilmington, MA). Mice with a targeted mutation of the *Raldh2* gene were as described [Niederreither et al., 1999]. Mice with a double mutation of retinol-binding protein and lecithin:retinol acyltransferase (*RBP/LRAT*) were previously described [Kim et al., 2008].

High-density culture

Otocysts with associated periotic mesenchyme were derived from the embryonic offspring of CD-1 mice. Cells were dissociated with 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA) and cultured according to standard procedures [Frenz and Van De Water, 1991]. Briefly, mesenchymal cells were resuspended in Ham's F-12 culture medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at a density of 2.5×10^7 cells/ml. Equivalent amounts of otic epithelial tissue per culture were mixed into the cell suspension, and 10- μ l droplets of cell suspension were plated in the centers of wells of a 4-well tissue culture plate (Nunc, Naperville, IL). After a 1 hour incubation at 37°C, 1 ml Ham's F-12 culture medium plus 10% FBS was added to each well. To test the rescue capabilities of FGF under aberrant RA conditions, some cultures were treated daily with 10^{-6} M RA (Sigma, St. Louis, MO) or with antisense oligonucleotides complementary to the mouse *Raldh2* or *RAR α* gene (150 μ g/ml), then supplemented after 24 hours in vitro with a combination of FGF3 and FGF10 (R & D Systems Inc., Minneapolis, MN) (15 ng/ml each; 48 hours). The oligonucleotides were prepared as purified products by Invitrogen and were as follows: *RAR α* , as described [Liu et al., 2008]; *Raldh2*, antisense, 5'-CTGCAGCGAGGCCAT-3'.

Organotypic culture

Organotypic cultures were prepared based on the method of Van De Water and Ruben [1974]. Briefly, otocysts with surrounding mesenchyme were explanted from CD-1 mouse embryos at embryonic age 10.5 days (E10.5) and placed individually on Millicell-CM inserts (Millipore, Billerica, MA) in 4-well plates containing 1 ml of Dulbecco's Modified Eagle Medium (DMEM) with glucose (Invitrogen) and 10% FBS. Explants were incubated in a H₂O-saturated atmosphere, and the culture medium was exchanged daily. Some explants were treated with pharmacological reagents beginning on the initial day of culture for a period of 48 or 72 hours. The reagents were as follows: citral (Sigma) (200 μ M, 300 μ M or 500 μ M), RAR antagonist Ro 41-5253 (Biomol, Plymouth Meeting, PA) (10 μ g/ml or 20 μ g/ml), or 4-diethylaminobenzaldehyde (DEAB) (Sigma) (15 μ g/ml or 30 μ g/ml). For rescue studies, a non-teratogenic dose of 10^{-8} M RA was added to the reagent-treated cultures.

Maternal administration of RA

RA was administered to gravid CD-1 mice following standard procedures [Frenz et al., 1996]. In brief, a solution of all-trans RA (Sigma) was made as 5 mg crystalline RA in 0.8 ml of absolute alcohol in 9.2 ml sesame oil and stored in the dark at 4°C. Gravid mice were administered 2 consecutive doses of 25 mg/kg RA by feeding needle at 10:30 AM and 2:30 PM on E9. Control mice received equivalent doses of the vehicle (i.e., alcohol in sesame oil) at corresponding times or were untreated.

Reverse Transcription (RT)-PCR

Total RNA was isolated (RNassay Kit, Qiagen, Valencia, CA) from mouse otocysts or cultures, and reverse-transcribed to first-strand cDNA using the SuperScript Preamplification System (Invitrogen). The second DNA strand was synthesized using *Taq* PCR Core Kit (Qiagen) and oligomers specific for the gene of interest. RT-PCR was performed as previously described [Liu et al., 2008]. The oligomers were as follows: *Dlx6*, forward-5'-GCTGAAGCAGGGTAGTAAC-3' and reverse-5'-CCTATCAGTCCTCAGATTGG-3'; *Gata3*, forward-5'-GAGTCTCAAGTGTGCGAAGAGT-3' and reverse-5'-TCGGGCTTCATGATACTGCTC-3'; *Wnt1*, forward-5'-CTGGGTTTCTACTACGTTGC-3' and reverse-3'-GTTCTGTCTGGATCAGTCG-3'; *Gbx2*, forward-5'-ACGGCTCGCTGCTCGCTTTC-3' and reverse-5'-GAGCTGTAATCCACATCGCT-3'; *GAPDH*, forward-5'-TGCACCACCAACTGCTTA-3' and reverse-5'-GGATGCAGGGATGATGTTTC-3'. Oligomers for *FGF3*, *FGF10*, *Dlx5*, β -catenin, and β -actin were as described [Liu et al., 2008].

Whole-mount in situ hybridization

Whole mount in situ hybridization was performed using the previously described protocol [Liu et al., 2008]. Embryos were dissected in cold phosphate buffered saline (PBS) and fixed overnight in 4% paraformaldehyde (PFA) in calcium/magnesium-free DEPC-PBS (4°C). The tissues were washed in PBS with 0.1% Tween-20, dehydrated, and stored at -20°C until use. Following rehydration, embryos were treated with proteinase K (10 mg/ml; 5 min), refixed in 4% PFA-0.2% glutaraldehyde (15 min), prehybridized (3 hr, 65°C), and incubated with hybridization mix, including antisense or sense digoxigenin (DIG)-labeled RNA probe at 70°C overnight. Anti-DIG-alkaline phosphatase conjugate (Boehringer-Mannheim, Palo Alto, CA) was used to detect the probe. Color was developed with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Embryos were washed 3 times in PBS with 1% Triton X-100 to stop the staining reaction.

Probe preparation

Dlx5-encoding plasmid, provided by Dr. Michael Shen, was as described [Liu et al., 2008]. Plasmid encoding *Dlx6* was a gift of Drs. M. Vieux-Rochas and Giovanni Levi. *FGF3*-encoding plasmid was provided by Dr. Suzanne Mansour [Wright and Mansour, 2003]. *FGF10*-encoding plasmid was as described [Alvarez et al., 2003]. Plasmids were linearized and transcribed according to standard procedures. Probes were DIG-labeled.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed essentially as described [Cvekl et al. 1995, 1994]. ³²P-labeled double-stranded oligonucleotides corresponding to RA responsive elements (RAREs) in the 5.7kb *FGF3* inner ear enhancer [Powles et al., 2004] were incubated with recombinant RAR/RXR protein and nonspecific competitor poly(dI-dC). RAR β and RXR β recombinant proteins (Active Motif, Carlsbad, CA and Protein One, Rockville, MD, respectively) were mixed 1:1. Competition for protein binding was evaluated using oligonucleotides containing

consensus binding sites for RAREs, as described [Minucci et al., 1994]. Protein-DNA complexes were separated by electrophoresis (6% native polyacrylamide gel using 0.5X Tris-borate) followed by autoradiography. Exposure time was 18 hours. The oligonucleotides were as follows: site “a”, 5'-GGCCTAGGGTCACATGGAGGCCCTGCTG-3'; site “b”, 5'-GCTGGCTTCAGCAGGTGGCCTTGGGAC-3'; RARE, 5'-GATCCGCTAGCAAGGGTTACCGAAAGCACTCGCATA-3'.

Cell transfection

Three copies of AP1/Ets modules (shown in Figure 12) were cloned 5' of the E4TATA minimal promoter in the luciferase vector pGL3 (Promega, Madison, WI) and transfected into MCF7 cells. Empty vector with E4TATA was used to control for induction by FGF via specific FGF responsive elements rather than the E4TATA fragment. Cells were cultured in 10 cm plates, and at 80–90% confluence, cells were passed into 24-well plates to achieve 50–60% confluence in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) without antibiotics. Sixteen hours later, when 80% confluence was reached, the cells were transfected (Invitrogen Lipofectamine 2000) with 0.8 µg of firefly luciferase reporter DNA for 4 hours, after which the culture medium was changed. An internal control plasmid Renilla luciferase encoding pRL-TK (Promega) was included. Twenty-four hours post transfection, cells were treated or not treated with FGF2 (100 ng/µl) and heparin (25 µg/µl) (18 hours). FGF2 was selected because the responsiveness of the MCF7 cell line to FGF2 has been established. The same downstream effectors of the FGF/MAPK arm come into play regardless of which FGF receptor and ligand is active. Cells were passively lysed, and luciferase activity measured at room temperature with a dual luciferase reporter assay kit (Promega). Firefly luciferase activities were normalized relative to Renilla luciferase activity.

WHAT BASIC PRINCIPLES UNDERLIE INNER EAR DEVELOPMENT THAT MAY BE PERTURBED BY ABERRANT RA SIGNALLING?

Formation of the inner ear is a multistep process that includes a series of sequential inductive tissue interactions. The mouse inner ear initially becomes evident at around 8 days of embryonic development (E8), when cephalic surface ectoderm interacts with signals coming from the neuroepithelium of the developing hindbrain to form a localized thickening known as the otic placode (Fig 1A) [Kiernan et al., 2002]. The otic placode thickens and invaginates to form an otic cup (E9) (Fig 1B) which by E9.5, closes and sinks below the surface to form an otic vesicle.

A second inductive event occurs when the epithelium of the otic vesicle interacts with surrounding periotic mesenchyme, promoting subsequent morphogenesis of the complex three-dimensional structure of the inner ear. The cochlear and vestibular anlagen of the inner ear (i.e. hearing and balance primordia) originate from the otic epithelium itself, whereas the cartilaginous otic capsule arises from the surrounding periotic mesenchyme. Retinoic acid is present in both the developing hindbrain adjacent to the developing inner ear, as well as the epithelium of the inner ear, and plays a role during each of these inductive phases of otic development [Romand et al, 2006]. Both excess and deficiency in retinoic acid prior to hindbrain segmentation (E7.75-E8) profoundly affect hindbrain development, leading to loss of rhombomeres (r4-r7) [White et al., 2000] or altered rhombomeric patterning [Gale et al., 1999; Marshall et al., 1992; Morriss-Kay et al., 1991]. This perturbation in hindbrain development at E7.75-E8 correlates with inner ear defects, and presumably reflects indirect effects of RA on the developing inner ear. However, maternal administration of RA subsequent to hindbrain patterning, at E9, impacts on patterning and morphogenesis of the

inner ear putatively due to direct affects of RA [Frenz and Liu, 2000; Garritano and Frenz, 2008].

HOW DOES RA INFLUENCE THE DEVELOPING INNER EAR? – Direct vs indirect effects

The enzymes that generate RA and the RAR/RXR receptors that transduce the RA signal represent the key molecular components that initiate the RA signaling pathway, and intriguingly show temporally and spatially restricted patterns of expression in the inner ear and adjacent tissues. This may reflect multiple modes of RA action on inner ear development (Fig 2). Retinaldehyde dehydrogenase 2 (Raldh2), a major retinoic acid synthesizing enzyme in the early embryo, is abundantly expressed in somitic mesoderm that neighbors the developing hindbrain [Haselbeck et al., 1999]. RA synthesized in the somitic mesoderm [Berggren et al., 1999; Molotkova et al., 2005] diffuses toward and regulates gene expression in the posterior hindbrain, even prior to the overt segmentation of rhombomeres [Romand et al., 2006], and in this way indirectly influences early development of the inner ear. A direct effect of somitic RA on the early otocyst is also likely to play some role (Fig 2) [Glover et al., 2006; Romand et al., 2006]. However, the expression of the RA synthetic enzymes (Raldh1, Raldh2, Raldh3) and retinoic acid receptors (RAR α , RAR β) within the otic epithelium itself [Romand et al., 2006, 2002] supports the contention that RA synthesized in the otic epithelium may act directly and locally on this tissue to control gene expression and thus regulate some aspects of its normal development [Garritano and Frenz, 2008]. Furthermore, since RAR α , β , and particularly γ are expressed in periotic mesenchyme, RA synthesized by the Raldh enzymes in the otic epithelium may act by diffusion on the surrounding mesenchyme to influence inner ear development [Romand et al., 2006] by means of a reciprocal interaction.

MOLECULAR MECHANISMS OF TERATOGENICITY - What signaling pathways are affected by exposure to excess RA?

Retinoic acid signaling is likely to mediate its effects by modulating other signaling pathways that regulate inner ear development [Garritano and Frenz, 2008]. The fibroblast growth factors (FGFs) comprise a family of signaling molecules that regulate cell proliferation, differentiation and migration during embryogenesis [Ornitz and Itoh, 2001] and are essential for appropriate patterning and formation of the developing inner ear [Alvarez et al., 2003; Wright and Mansour, 2003]. Examination of potential interactions between the FGF pathway and RA signaling was prompted by studies which show that loss of either *FGF3* or *FGF10* leads to inner ear anomalies that include some defects reminiscent of those caused by exposure to excess RA. These anomalies include failure of endolymphatic duct formation and reduced cochlear coiling in the *FGF3* null mutant inner ear [Mansour et al., 1993; Hatch et al., 2007] and agenesis or reduction of the semicircular canals in the *FGF10* null mutant inner ear [Pauley et al., 2003]. Embryos lacking both *FGF3* and *FGF10* fail to form otic vesicles [Wright and Mansour, 2003] or form severely reduced vesicles [Alvarez et al., 2003]. Of significance, mutations of *FGF3* and of *FGF10* have been associated with human deafness. Major phenotypic effects, including profound congenital sensorineural deafness, complete labyrinthine aplasia (Michel aplasia) and microtia have been noted in patients with homozygous mutations in the *FGF3* gene [Tekin et al., 2007, 2008]. Heterozygous mutations in the genes encoding the tyrosine kinase domains of *FGF* receptors 2 and 3, and a mutation in *FGF10* were identified in lacrimo-auriculo-dento-digital (LADD) syndrome, which besides affecting the nasolacrimal ducts, teeth and salivary glands, is characterized by external ear anomalies and quite frequently with sensorineural, conductive, or mixed-type hearing loss [Rohmann et al., 2006]. These defects mimic a

number of the clinical manifestations of excess RA exposure during the first trimester of pregnancy (e.g. Michel aplasia, sensorineural deafness, microtia) [Lammer, 1985] and are reminiscent of the otic anomalies associated with aberrant RA levels in animal models. In each case, members of the *FGF* signaling pathway (*FGF3*, *FGF10*) are affected.

Expression of *FGF3* and *FGF10* mRNA coincides both spatially and temporally with formation of the mouse inner ear (Figure 3), being expressed in the otic cup and subsequently in the presumptive cochlear and vestibular sensory epithelium [Ohuchi et al., 2005; Pauley et al., 2003; Pirvola et al., 2000]. *FGF10* transcripts, initially detected in the mesenchyme underlying the presumptive otic ectoderm (1–7 somite stage), become diminished in the mesenchyme by E8.75 and restricted to the otic epithelium [Alvarez et al., 2003; Pirvola et al., 2000]. Genes encoding *FGF3* and *FGF10* are also expressed in the developing hindbrain next to the region where the otic placode and vesicle develop (Fig 3) [Zelarayan et al., 2007; Ohuchi et al., 2005; Alvarez et al., 2003; Pirvola et al., 2000]. It has thus generally been accepted that an effect of excess RA on the inner ear is a secondary consequence due to changes in *FGF* signaling in the hindbrain [Hans and Westerfield, 2007] in a related manner to the way the hindbrain Hox code is altered. However, when excess RA is administered to gravid mice at E9, a timepoint at which hindbrain segmentation and marker expression are not altered by RA (Frenz, unpublished result), expression of *FGF3* and *FGF10* mRNA are both downregulated within the epithelium of the otocyst [Liu et al., 2008]. This diminution in *FGF3* and *FGF10* expression by RA is noted at E9.5 and as early as 7 hours post RA administration (Fig 4), alluding to a direct effect of RA on *FGF* signaling. RA-treated embryos with no otic defects do not show changes in *FGF3* expression. Importantly, excess RA exposure at E9 does not modify expression of a number of other key transcription factors (*Gbx2*, *Gata3*) and signaling pathways (*Wnt1* and β -catenin) (Fig 4). While this certainly does not preclude contributions from other signaling molecules in RA-mediated teratogenicity, it does support RA specificity. Furthermore, the dysregulation of only a number of inner ear genes argues against a catastrophic effect of non-homeostatic concentrations of RA as the explanation for otic dysmorphogenesis.

The Goldilocks phenomenon - Does deficiency in RA also affect FGF signaling?

RA has been shown to act as a morphogen in the neural tube, where specific concentrations of RA either upregulate or downregulate particular transcription factors (e.g. Hox genes, class I/II homeodomain genes) to pattern the neural tube in two dimensions [Briscoe and Ericson, 2001]. RA functions within optimal concentration ranges, reminiscent of the classic children's story "Goldilocks and the Three Bears", where the temperature of the porridge can be neither too hot or too cold, but "just right". Thus it was predicted that if excess RA has an effect on *FGF* signaling in the inner ear, deviations in the opposite direction, i.e. deficiency in endogenous RA, would also likely produce changes in *FGF* signaling and lead to otic dysmorphogenesis. This has been addressed using loss-of-function mouse mutations to mimic conditions of RA deficiency. Since there is no de novo fetal synthesis of vitamin A in the mammalian embryo, developing tissues are strongly reliant on maternal circulating retinoids that reach the embryo by means of the placenta [Kim et al., 2008; Marceau et al., 2007]. Double mutation of retinol-binding protein (*RBP*) and lecithin:retinol acyltransferase (*LRAT*) [Kim et al., 2008], which are respectively responsible for retinol transport in the circulation and formation of retinyl esters, the storage form of vitamin A, leads to severe retinoid deficiency under conditions of maternal dietary vitamin A deprivation [Kim et al., 2008]. In *RBP/LRAT* double null mutant embryos, loss of *FGF3* and *FGF10* expression was noted (Fig 5).

Deficiency in RA biosynthesis, e.g. in *Raldh2* null embryos, would also be expected to alter expression of FGFs, particularly since the *Raldh2* null otocyst is hypoplastic and abnormally distant from the hindbrain neuroepithelium [Niederreither et al., 1999], reminiscent of the

vitamin A deficient phenotype. However, targeted disruption of *Raldh2* also perturbs early neural tube development with defects in segmentation of the hindbrain, resulting in only weak and inappropriately restricted expression of *FGF3* in r5/r6 [Niederreither et al., 1999]. The hindbrain is an important signaling center for inner ear induction (see introduction), and does so via FGF signaling, and thus it remains a challenge to distinguish whether the resultant change in otic phenotype in this model is due to direct or indirect effects of RA on *FGF* signaling in the inner ear. In addition, these animals (*Raldh2* null mutant) are embryonic lethal, precluding assessment of the inner ear and its gene expression patterns subsequent to E9.5-E10.

In order to circumvent these difficulties, an in vitro model was developed in which the otocyst and surrounding mesenchyme are explanted at E10.5, subsequent to the timepoint when patterning of the hindbrain is already established, and explants can be maintained in the presence or absence of pharmacological reagents which block RA signaling. These reagents include RA receptor (RAR) antagonist Ro 41-5253 (Biomol), and citral and 4-diethylaminobenzaldehyde (DEAB) (Sigma), which inhibit aldehyde dehydrogenases and block the conversion of retinol to retinoic acid as a substrate for *Raldh* [Chute et al., 2006; Tanaka et al., 1996]. Treatment of explants with these reagents at E10.5, thus after the time during which the hindbrain influences otic development, leads to the formation of small hypomorphic otocysts which recapitulate the otocyst phenotype of vitamin A deficient mouse embryos (Fig 6). In addition, pharmacologic blocking leads to diminished levels of *FGF3* and *FGF10* expression, but not other markers of otic development (Fig 5), further supporting a direct effect of RA on *FGF* signaling in the inner ear.

It was predicted that if any reduction of *FGF* in these explants is directly due to blocking of RA, *FGF* expression should be rescued by an appropriate concentration of exogenously added RA. To test this, otic explants were first treated with DEAB then supplemented with a non-teratogenic dose of RA (10^{-8}M). To control for the rescue, *FGF* expression was also analyzed in explants where RA receptors are antagonized. Rescue of *FGF3* and *FGF10* by RA was achieved in DEAB-treated explants, but not in explants treated with the RAR antagonist Ro 41-5253 (Fig 7). Importantly, 10^{-8}M RA is not sufficient to alter *FGF* expression in control explants (Fig 7), suggesting the exogenous RA is necessary and sufficient for the DEAB-treated explant rescue.

Control of FGF signaling by RA

Little is known about how retinoid signals are interpreted at the genetic level. However, deciphering the mechanisms for transcriptional regulation of *FGFs* by RA is critical to understand how RA mediates its control of inner ear development. In earlier studies, a transgenic approach was used to assay genomic regions from the mouse *FGF3* gene for regulatory activity. A minimal enhancer of 5.7 kb from the mouse *FGF3* locus was identified that governs expression in the inner ear, but not in the body of rhombomeres 5/6 [Powles et al., 2004]. Sequence analysis of the 5.7 kb minimal inner ear *FGF3* enhancer indicates the presence of putative DNA binding sites where retinoid signaling could directly mediate its effects. We identified two putative retinoic acid responsive elements (RAREs) within the enhancer (site a, site b) and performed an electrophoretic mobility shift assay (EMSA) in which oligonucleotides encompassing these sites were tested for their ability to bind RAR β /RXR β heterodimer proteins (Fig 8). Our findings reveal specific binding of a predicted site by recombinant RAR β /RXR β , which was abolished by excess cold RARE [Minucci et al., 1994], providing proof-of-principle of direct regulation of *FGF* by RA.

Downstream targets of FGF are altered by deviations in RA

To further understand molecular mechanisms by which disruption in *FGF* signaling by aberrant levels of RA may lead to otic defects, downstream targets of *FGF* were examined under conditions of RA excess and deficiency. *Dlx5* and *Dlx6*, members of a highly conserved family of homeobox genes [Merlo et al., 2000], are expressed in the epithelium of the developing otocyst [Robledo and Lufkin, 2006; Merlo et al., 2002] and downregulated in mutant mice by targeted mutation of the *FGF3* and/or *FGF10* gene [Wright and Mansour, 2003] (Frenz, unpublished result). Recent studies show that *Dlx5* and *Dlx6* are also downregulated in otocysts in mouse embryos exposed in utero to excess RA (Fig 9) [Liu et al., 2008]. This change in *Dlx5/Dlx6* expression was noted at E10.5 days, but not at the earlier timepoints (e.g. E9.5) when changes in *FGF3* and *FGF10* expression have already occurred (Fig 9). It is thus likely that *Dlx5* and *Dlx6* are not direct RA target genes. In support of this, sequence analysis of related *Dlx* genes (i.e. *Dlx3* and *Dlx4*) reveals elements that resemble RA responsive elements (RAREs) but none that match exactly [Ellies et al., 1997], while the 5' flanking region of *Xenopus Dlx2* does not contain sequences that match consensus RAREs [Studer et al., 1994].

Similar to conditions of RA excess, reduction in *Dlx5* and *Dlx6* expression also occurs in retinoid-deficient otocysts in mouse embryos carrying a double mutation of *RBP* and *LRAT* (Fig 10). Accordingly, when *Raldh2* mutant embryos are treated with limited maternal RA to levels just sufficient to promote viability, expression of *Dlx5* is reduced in the RA-deficient *Raldh2* null inner ear (Fig 10). This diminution is noted at E10.5, but appears normal at E9.5, suggesting that compromised *Dlx5* expression is affected during a defined developmental window in which retinoid intake is either limited or excessive. Treatment of otic explants with DEAB or Ro 41-5253 (RAR antagonist) also leads to downregulation of *Dlx5* and *Dlx6* expression that is independent from the influence of the hindbrain (Frenz, unpublished result). Furthermore, when otic cultures are treated either with antisense oligonucleotides complementary to the mouse *Raldh2* or *RARα* gene to model conditions of RA deficiency, or with excess RA, levels of *Dlx5* and *Dlx6* are diminished but can be restored by supplementation with FGF3 and FGF10 (Fig 11). Collectively, these data confirm the importance of maintaining a critical level of endogenous RA for appropriate regulation of target gene expression in inner ear development, and lend well to the hypothesis that reduction in *Dlx5* and *Dlx6* expression due to aberrant levels of RA may occur as a secondary consequence to changes in *FGF* signaling. This hypothesis is particularly intriguing, given that deletions that include *Dlx5* and *Dlx6* on chromosome 7q21 are associated with syndromic split hand/split foot malformation, which is sometimes accompanied by sensorineural hearing loss [Tzschach et al., 2007; Haberlandt et al., 2001], Mondini dysplasia [Wieland et al., 2004] and other inner and middle ear anomalies that resemble retinoid embryopathies, i.e., hypoplasia of the cochlea, malformations of the malleus and incus, and anomalies affecting the semicircular canals [Fukushima et al., 2003].

Evidence for direct regulation of *Dlx* by FGF

We have begun to explore the possibility that expression of *Dlx5/Dlx6* may be directly regulated by *FGF* signaling. Several signaling pathways can be activated by *FGF* signaling to modulate regulation of gene expression. The best understood FGF-initiated pathway is the MAP kinase (MAPK) pathway [Bottcher and Niehrs, 2005; Chang and Karin, 2001], which is active in inner ear development [Urness et al., 2008]. The MAPK signaling cascade terminates with de novo transcription [Barolo and Posakony, 2002] or phosphorylation of nuclear transcription factors comprising the AP-1 [Karin, 1995] and Ets [Sharrocks, 2001] families. Using the UCSC genome browser, we determined that there are evolutionary conserved regions within ~10 kb between *Dlx5* and *Dlx6* in which potential FGF-responsive AP-1 and Ets sites have been identified (Fig 12). We generated a firefly luciferase reporter

driven by the E4TATA minimal promoter together with candidate FGF-responsive elements (FREs) from this region and assayed reporter expression in the presence or absence of FGF2 in transient transfections. Empty luciferase vector pGL3 with E4TATA was used as a control to confirm that induction by FGF2 is via specific FREs rather than the E4TATA fragment. Our findings have thus far demonstrated a two-fold induction by FGF2 when the reporter construct was tested in the MCF7 cell line (Fig 12). This data supports the contention that the identified *Dlx5/Dlx6* regulatory region contains at least one “short” FGF-responsive element. Remaining elements will be identified in future studies.

How changes in FGF/Dlx signaling lead to otic dysmorphogenesis

A *Dlx5* mutation was used as a means to better understand how changes in *FGF-Dlx* signaling in the otic epithelium can underlie RA-mediated inner ear teratogenesis [Liu et al., 2008]. Inductive signals emanating from the otic epithelium act on the surrounding periotic mesenchyme to promote formation of the cartilaginous otic capsule, which is dysmorphic in both RA-exposed and *Dlx5* null mutant embryos [Merlo et al., 2002; Acampora et al., 1999]. Otic epithelium is able to induce cartilage formation in explants but previous studies showed that otic epithelium derived from embryos exposed in utero to excess RA is unable to effectively induce chondrogenesis in cultured periotic mesenchyme [Frenz and Liu, 2000]. One hypothesis to account for this envisages aberrant otic epithelium signaling following RA treatment, with the loss of chondrogenesis in part caused by reduced *Dlx5* expression as a contributing factor. Otic capsule chondrogenesis was thus used as an assay to gauge how changes in *Dlx5* gene expression in the otic epithelium can cause qualitative differences in the surrounding mesenchyme [Liu et al., 2008], differences that underlie otic capsule defects seen in *Dlx5* mutant mice [Depew et al., 1999]. Otic epithelium derived from *Dlx5* null mutant embryos was tested in our high-density culture model [Frenz and Van De Water, 1991] for its ability to induce chondrogenesis in periotic mesenchyme harvested from control embryos. Mesenchymal condensations, which presage the formation of cartilage, were counted in each culture and used as an early index of chondrogenesis. *Dlx5* null otic epithelium was not as effective in evoking a chondrogenic response as was otic epithelium harvested from a combination of *Dlx5* wild-type and heterozygous embryos (Table I). We suggest that a similar situation, in which the inductive ability of otic epithelium is compromised by *Dlx* deficiency, is operant under conditions of aberrant RA.

This raises the question as to how altered expression of *Dlx5*, present only within the otic epithelium, elicits non-cell autonomous effects on the surrounding periotic mesenchyme. This might be illuminated by recent studies which show that expression of transforming growth factor-beta ($TGF\beta_1$), a signaling molecule that mediates chondrogenic differentiation of periotic mesenchyme [Frenz et al., 1992], is markedly diminished within the mesenchyme of the forming capsule in the *Dlx5* null mutant inner ear in comparison to wild-type littermates [Liu et al., 2008]. Similarly, $TGF\beta_1$ is downregulated in the capsule (periotic) mesenchyme of the in utero RA-exposed inner ear [Frenz and Liu, 1997]. Other signaling molecules that contribute to otic capsule chondrogenesis, including *Wnt5a*, *Ihh* and *FGF2*, are not affected by the *Dlx5* mutation (Frenz, unpublished result). Furthermore, when added to cultures comprised of mesenchyme and epithelium derived from *Dlx5* null inner ears, $TGF\beta_1$ is able to restore chondrogenesis to normal levels [Liu et al., 2008]. Thus loss of *Dlx5* expression, either directly due to targeted inactivation of the gene, or indirectly due to aberrant RA exposure (overexpression or deficiency) via the effects of RA on *FGF* signaling, may affect regulation of the chondrogenic program in periotic mesenchyme. Perhaps then, *Dlx5* expression initiates a second signaling cascade from the otic epithelium to initiate the chondrogenic program and $TGF\beta$ expression in this nearby periotic mesenchyme. The mechanism of how epithelial *Dlx5* expression modulates periotic $TGF\beta$

expression will be particularly interesting as will underpinning this key inductive event required for otic capsule formation (Fig 13).

CONCLUSION

There is a critical developmental period in which morphogenesis of the inner ear is dependent upon endogenous retinoid signaling. Conditions which produce non-optimal RA levels, either in excess or deficiency, lead to defects in inner ear development. These defects can result by the aberrant RA downregulation of *FGF3* and *FGF10* expression, which in turn modifies expression of downstream target genes (*Dlx5*, *Dlx6*). We propose a model in which local synthesis of RA in the otic epithelium initiates a regulatory cascade that coordinates aspects of inner ear morphogenesis, such as capsule development, by controlling *FGF/Dlx* signaling. The next key step is to decipher the mechanisms for transcriptional regulation of *FGF* by RA, and thus unravel the genetic pathways linking endogenous RA production in the otocyst and the program of inner ear morphogenesis.

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Figure 1.

Scanning electron micrograph of left, an E8.5 mouse embryo, showing development of a bilateral otic placode (arrows) from cephalic surface ectoderm (SE), just caudal to the first branchial arch (1) and lateral to the fusing edges of the rhombencephalon (R); and right, the otic cup (OC) of an E9 mouse embryo. Periotic mesenchyme (M) surrounds the epithelium of the otic cup. Arrows demarcate the dorso-lateral boundary of the invaginating otic cup. Permission for reprint requested from Excerpta Medica.

Figure 2.

Schematic depicting the influences of endogenous RA on the developing inner ear. RA synthesized by Raldhs in the somitic mesoderm (So) diffuses toward the developing hindbrain where it can influence *FGF* gene expression, and indirectly affect the otocyst (Ot). It is also possible that RA synthesized in the somitic mesoderm may diffuse toward the developing otocyst. Our studies suggest that RA synthesized in the otic epithelium, evidenced by the expression of Raldhs, can directly affect the otic epithelium and modify gene expression (e.g. *FGF*) within this tissue. Oe, otic epithelium; M, periotic mesenchyme; RA, retinoic acid; r, rhombomere. Adapted with permission from Wiley & Sons, Inc. [Romand et al., 2006].

Figure 3.

Expression of *FGF3* and *FGF10* coincides spatially and temporally with formation of the inner ear. A. Whole mount in situ hybridization showing *FGF3* expression in the otic vesicle (OV) and developing vestibulo-acoustic ganglia (gVIII) at E10.25. B. Horizontal serial section reveals *FGF3* expression in the anterior lateral wall of the otic vesicle and ganglia (gVIII). C. By E9, *FGF10* expression is noted in the otic cup (whole mount in situ hybridization). D. Transverse section (indicated by a line in C) demonstrates expression of *FGF10* in the otic cup (oc) and pharyngeal endoderm (pe). E. Whole mount in situ hybridization of an E9.5 mouse embryo reveals that *FGF3* is expressed in rhombomeres 5 and 6 adjacent to the area where the otic vesicle will form. F. Double in situ hybridization

for *FGF10* and *Mafb* (red), a marker expressed at the level of r5 and r6, shows expression of FGF10 in these rhombomeres at E9. Reprinted with permission from Wiley & Sons, Inc (A, B, E, F) and the Company of Biologists, Ltd (C, D).

Figure 4.

A, B) Whole mount in situ hybridization of RA-exposed (B) and control (A) embryos, showing a diminution in *FGF10* in the otocyst (demarcated by arrows) of the RA-exposed embryo in comparison to the control embryo. C-J) RT-PCR analysis confirmed these findings, demonstrating a change in *FGF3* and *FGF10* in response to in utero RA exposure but not other markers of otic development. C) A reduction in *FGF10* is evident in RA-exposed otocysts at E10.5 (lane 2) and E9.5 (lane 4) in comparison to their respective controls (lanes 1 and 3). Lane 5, RA-exposed embryo (no otocysts). D) Analysis of *FGF10* at various timepoints post RA administration shows *FGF10* diminution in the otocysts of malformed embryos at E9.5 (lane 1), E9 (lanes 2,4) and as early as 18, 15 and 7 hours post RA administration (lanes 6, 7 and 5 respectively) in comparison to nonmalformed control embryos (lane 3). E) Comparison of lane 1 and lane 2 shows downregulation of *FGF3* in RA-exposed otocysts (lane 2) at E9.5 days compared to control otocysts (lane 1). F) β -actin controls on the templates of E9.5 and E10.5 otocysts (lanes 1 and 3, control; lanes 2 and 4, RA-exposed) corresponding to lanes 1–4 in (C), lanes 1–2 in (E), lanes 1–4 in (G), lanes 1–2 in (H), lanes 1–4 in (I), lanes 1–4 in (J), and lanes 1 and 3 in (D). β -actin controls corresponding to each sample in lanes 2, 4, 5–7 in (D) performed (but not shown). G-J) Exposure to RA does not alter expression of β -catenin (G; lane 1, 2 - E10.5 control, RA-exposed; lane 3, 4 - E9.5 control, RA-exposed), *Gata3* (H; lane 1, 2 - E10.5 control, RA-exposed), *Wnt 1* (I; lane 1, 2 - E10.5 control, RA-exposed; lane 3, 4 - E9.5 control, RA-exposed) and *Gbx2* (J; lane 1, 2 - E9.5 control, RA-exposed; lane 3, 4 - E10.5 control, RA-exposed).

Figure 5. Whole-mount in situ hybridization for *FGF3* in mice with a double mutation of *LRAT* and *RBP* [Kim et al., 2008], showing diminished expression of *FGF3* in the developing otocyst (demarcated by arrows) of the null mutant embryo (left) in comparison to its wild-type littermate (right).

Figure 6.

Blockade of RA signaling by pharmacological reagents. A–E) Organotypic cultures of the inner ear [Van De Water and Ruben, 1974], in which RA deficiency was modeled by treatment with citral, Ro 41-5253 or DEAB (72 hours in vitro). A) Control otocyst (demarcated by arrows) showing outpocketing of the developing endolymphatic duct and sac anlagen (ED), forming cochlear duct (CD) and statoacoustic ganglion (SG). B,C) Citral-treated otic explants (200 and 500 μM respectively) showing a more marked perturbation in otocyst development with increasing concentration of citral. D, E) RAR antagonist Ro 41-5253- (D; 10 μg/ml) and DEAB- (E; 30 μg/ml) treated explants show similar perturbation in otocyst development. F–N) RT-PCR analysis demonstrating changes in *FGF* expression, but not other markers of otic development, following treatment with pharmacological reagents. F) *FGF10* and G) *FGF3* – lane 1, control; lane 2, citral, 200 μM; lane 3, citral, 300 μM. H) *FGF3* – lane 1, control; lane 2, DEAB (15 μg/ml). I, J, K) *β-actin* controls corresponding to F, G, and H respectively. L) *Gata3* – lane 1, control; lane 2, citral, 200 μM (48 hrs); lane 3, citral, 300 μM (48 hrs); lane 4, control; lane 5, DEAB (15 μg/ml; 48 hrs). M) *Gbx2*, lanes 1–5, as in (L); lane 6, control; lane 7, Ro 41-5253 (10 μg/ml; 48 hrs). N) Corresponding *β-actin* controls (L, M). Permission requested for reprint of A, B, C, F, I from Libertas Academica.

Figure 7.

Rescue of *FGF* by RA under RA-deficient conditions in vitro. RT-PCR analysis of otic explants showing A) diminished *FGF3* in otic explants treated with DEAB (30 $\mu\text{g/ml}$) (lane 2) in comparison to control explants (lane 1), and the ability of a non-teratogenic dose of RA (10^{-8}M) to restore levels of *FGF3* expression in DEAB-treated explants (lane 3). Treatment with 10^{-8}M RA alone did not significantly affect *FGF3* expression (lane 4). B) Corresponding GAPDH controls. C) To control for the rescue, otic explants were treated with the RAR antagonist Ro 41-5253 (20 $\mu\text{g/ml}$), then supplemented with 10^{-8}M RA. Treatment with Ro 41-5253 leads to reduced expression of *FGF10* (lane 2) in comparison to control explants (lane 1) however, supplementation of Ro 41-5253-treated otic explants with RA did not rescue *FGF10* expression (lane 3). RA (10^{-8}M) alone did not modify expression of *FGF10* (lane 4). D) Corresponding GAPDH controls.

Figure 8.

Identification of RAREs within 5.7kb enhancer. A) Genomic arrangements of the mouse *FGF3* locus. B) Prediction of RAREs in the 5.7 kb enhancer by searching the consensus motif. Upper panel: The RARE consensus sequence contains 2 hexanucleotide repeats (direct or palindromic) and a variable linker of 1–10 nucleotides (Chambon, 1996). Middle panel: DR3 sequence logo is a representative binding motif identified by protein-DNA binding selection assay JASPAR database: http://mordor.cgb.ki.se/cgi-bin/jaspar2005/jaspar_db.pl. DR3: direct repeat with 3bp linker. Larger characters denote more conservation of a specific nucleotide at that position. Lower panel: Two predicted RAREs, site “a” at 4741 is a direct repeat sequence with 5bp linker, and site “b” at 4763 is a palindromic sequence with 5 bp linker. C) EMSA results: RARE probe (positive control) is from published RXR β binding sites (Minucci et al, 1994). Site “a” shows specific binding by RAR β /RXR β , which is abolished by excess cold RARE. RAR β and RXR β are recombinant proteins (Active Motif and Protein One respectively) and are mixed 1:1. Exposure time: 18 hrs.

Figure 9.

In utero exposure to excess RA modifies *Dlx5* expression. Whole mount in situ hybridization reveals that following maternal exposure to excess RA, *Dlx5* is downregulated in the developing otocyst (arrows) at E10.5 days (B), but not as early as E9.5 (A) when compared to control embryos. Magnified views of the otocysts are provided below their respective embryos. These findings were confirmed by RT-PCR analysis, which shows C) diminished levels of *Dlx5* expression at E10.5 in RA-exposed otocysts (lane 4) compared to control otocysts (lane 3), contrasted by comparable expression in RA-exposed (lane 2) and control (lane 1) otocysts at E9.5 days. D) Corresponding β -actin controls. Reprinted with permission from Wiley & Sons, Inc. [Liu et al., 2008].

Figure 10.

Whole mount in situ hybridization for *Dlx5* and/or *Dlx6* in *Raldh2* mutant (A–D) [Niederreither et al., 1999] and *LRAT/RBP* mutant (E–H) [Kim et al., 2008] embryos. Comparison of (A) to (B) shows notable differences in the size of the otocyst in *Raldh2* null mutant (A) versus wildtype (B) embryos, however, levels of expression of *Dlx5* are not remarkably altered. This is contrasted at E10.5, when levels of *Dlx5* expression are diminished in the developing inner ears of RA-deficient *Raldh2* null mutant embryos (D) compared to wildtype controls (C). Comparison of (E) to (F) and (G) to (H) shows a reduction in *Dlx5* (E,F) and *Dlx6* (G,H) expression in the otocysts of E10.5 *LRAT/RBP* null mutant embryos in comparison to their wildtype littermates.

Figure 11.

RT-PCR analysis showing rescue of *Dlx* expression by FGF. A, C). Cultures of periotic mesenchyme and otic epithelium were treated with excess RA (10^{-6} M; days 1–7), then supplemented on day 2 with a combination of FGF3 and FGF10 (30 ng/ml total; 48 hours). Expression of *Dlx5* (A; lane 3) and *Dlx6* (C; lane 3) is downregulated by treatment with RA compared to their respective control cultures (A, C; lane 1), but could be restored by supplementation with FGF3 and FGF10 (A, C; lane 2). B) Corresponding β -actin controls. D–G) The rescue capabilities of FGF3 and FGF10 were tested under RA deficient conditions. Cultured periotic mesenchyme and otic epithelium were treated with antisense oligonucleotides complementary to either the mouse *Raldh2* or *RAR α* gene (150 μ g/ml) to interfere with endogenous RA signaling. D) RT-PCR analysis showing that *FGF10* (and *FGF3*, not shown), but not β -catenin (E), is diminished by treatment with *Raldh2* antisense oligonucleotide. Expression of *Dlx6*, downregulated by *Raldh2* (E; lane 3) and *RAR α* (G; lane 2) antisense oligonucleotides, was restored to control levels (E, lane 1; G, lane 3) by supplementation with FGF3 and FGF10 (E, lane 2; G, lane 1). β -actin controls corresponding to each sample in D–G were performed (not shown).

Figure 12.

A) Nucleotide sequence of candidate AP-1 and Ets-binding sites from an evolutionary conserved linker region between mouse *Dlx5* and *Dlx6*. AP-1 sites (black box), Ets-core binding sites (red box), and FGF/MAPK-responsive regions (green box). B) Sequence alignments between consensus binding sites (CON) and candidate binding sites. The nucleotides flanking the Ets “core”, GGA(A/T), are different among Ets family members [Graves and Peterson, 1998]. The Ets-sites 152/148 (176/173) closely resemble functional sites in the SRF promoter (Ets-2 binding site in the PS-1 promoter), respectively. C. Candidate responsive elements (marked by arrow in A) were cloned into firefly luciferase vector with minimal E4TATA promoter (schematic) and transfected into MCF7 cells [Gopal-Srivastava et al., 1998]. The bar graph shows that a two-fold induction occurred in response to treatment with FGF2 (construct pQ077). Empty vector with E4TATA was used as a control (construct p256C).

Figure 13.

Schematic illustrating that during a critical developmental window, RA, either in excess (+ RA) or deficiency (– RA), can directly alter *FGF* (3, 10) expression in the otic epithelium. This in turn affects downstream *Dlx* (5, 6), whose expression is restricted to this tissue (otic epithelium). Loss of *Dlx* affects regulation of molecular markers in the adjacent periotic mesenchyme, including *TGFβ*, by signaling from the otic vesicle.

Table I**B. *Dlx5* mutant otic epithelium + control periotic mesenchyme^a**

Experiment	Epithelium	Mesenchyme	Condensation No. ^b
1	CBA C57/BL6	CBA C57/BL6 ^c	92
	<i>Dlx5</i> ^{+/-} ; +/+	<i>Dlx5</i> ^{+/-} ; +/+	88
	<i>Dlx5</i> ^{-/-}	<i>Dlx5</i> ^{+/-} ; +/+	17
2	CBA C57/BL6	CBA C57/BL6	87
	<i>Dlx5</i> ^{+/-} ; +/+	<i>Dlx5</i> ^{+/-} ; +/+	91
	<i>Dlx5</i> ^{-/-}	<i>Dlx5</i> ^{+/-} ; +/+	23
3	CBA C57/BL6	CBA C57/BL6	90
	<i>Dlx5</i> ^{+/-} ; +/+	<i>Dlx5</i> ^{+/-} ; +/+	86
	<i>Dlx5</i> ^{-/-}	<i>Dlx5</i> ^{+/-} ; +/+	14

^aOtic epithelium derived from *Dlx5* null (*Dlx5*^{-/-}) or a combination of *Dlx5* heterozygous and wildtype (*Dlx5*^{+/-};+/+) embryos was interacted in culture with periotic mesenchyme derived from *Dlx5*^{+/-};+/+ (control) embryos.

^bMesenchymal condensations, which presage the development of cartilage, were counted in each culture on day 3 and used as an early index of chondrogenesis.

^cCultures comprised of CBA C57/BL6 epithelium and mesenchyme controlled for the mutant cultures.

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