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Molecular signaling in retinoic acid-induced inner ear teratogenesis

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Introduction

The developing inner ear takes its origin from a simple otic vesicle that gives rise to the complex three dimensional organs of hearing and balance. This process is dependent first on inductive signals from mesoderm and hindbrain neuroectoderm, and subsequently on reciprocal interactions between otic epithelium and its surrounding periotic mesenchyme. Retinoic acid, a biologically active derivative of vitamin A, is essential to both these inductive morphogenetic events. However, the normal function of retinoic acid in guiding inner ear development is achieved only at homeostatic concentrations. Deviations from optimal retinoid levels, either in excess or deficiency, results in inner ear dysmorphogenesis.

Approximately one-third of the cases of hearing loss due to congenital inner ear malformations are attributed to non-hereditary factors, including those due to environmental factors, such as exposure to teratogenic agents, or in the case of low and middle income countries, inadequate nutrition. Retinoic acid falls into both these categories. Infants born to mothers exposed to oral retinoids during a critical period of development have a 25-fold increased risk for craniofacial malformations [1], while an estimated 124 million children are affected each year by vitamin A deficiency, increasing their risk for congenital anomalies [2,3,4]. Pathological effects produced in the inner ear by conditions of vitamin A deficiency or in utero exposure to excess retinoids (e.g. 13-cis-retinoic acid) [5,6,7] are associated with impaired inner ear functionality [8,9]. Maternal exposure to even topical tretinoin (all-trans retinoic acid) during the first trimester has been associated with otic anomalies [10].

Retinoid mediated inner ear embryopathies have been reproduced in animal models. Rats maintained on diets deficient in vitamin A, the source of cellular retinoic acid, results in the formation of immature and small otic vesicles typified by atrophy of the sensory epithelium and often caudal location [11]. In some cases, absence of the
otic vesicle ensues as a consequence of complete elimination of dietary vitamin A. Vitamin A deficient quails are characterized by disrupted otocyst development [12], while mice harboring mutations in retinoid receptors or metabolizing enzymes that lead to deficiencies in endogenous retinoid signaling exhibit anomalies of the developing inner ear that recapitulate vitamin A deficiency [13,14,15,16,17]. Excess retinoic acid is just as harmful for development. At high doses, maternally administered retinoic acid gives rise to abnormal vestibular and cochlear development in the inner ears of mouse, rat and hamster embryos [18,19,20]. In zebrafish, excess retinoic acid results in the formation of ectopic and supernumerary otic vesicles [21].

The teratogenicity of retinoid excess and deficiency underscores the importance of regulating local retinoid concentrations during inner ear development. This review provides an overview of the implications of retinoic acid signaling during inner ear development, the pathways that mediate its effects and the consequences that ensue to critical signaling molecules when retinoid levels deviate from normal, either in excess or deficiency.

**Stage-dependent effects of retinoids**

Teratogenic effects of retinoic acid occur at several susceptible phases of embryogenesis [22]. The gamut of otic malformations resulting from exposure to retinoic acid is critically dependent on both the dose of retinoic acid administered and the developmental stage during which the otic primordia are exposed to excess retinoids [18,20,22]. This may be due to differential sensitivities of otic structures to retinoids during their formation ([22]. Although the focus of this review is the inner ear, a brief overview of how changes in retinoic acid signaling at slightly different stages of development dramatically affect different target tissues in the ear is discussed below.
Middle ear anomalies affecting the malleus, incus, stapes and tympanic ring, for example, are produced by exposure of embryonic mice to retinoic acid within a narrow temporal framework between embryonic age 8.5 to 8.75 days (E8.5 to E8.75) (Figure 1) [23,24,25]. This is the window of time during which cranial neural crest cells reach the first branchial arch ([23], from which middle ear structures originate [26]. Similar anomalies of the ossicles are observed in conjunction with defective formation of Meckel’s cartilage when gravid rats are administered retinoids at comparable stages of development [27,28]. Histological studies of the temporal bone in affected humans are limited, but show similarities to the teratogenetically induced defects in the mouse and rat embryos. Examination of human fetuses born to mothers receiving isotretinoin before and/or during the first weeks of gestation demonstrate a slit-like tympanic cavity, anteriorly shifted or malformed incus, medially displaced or anomalous malleus, and deformities of the stapes [7,29]. A hypoplastic facial nerve and absence of the chorda tympani nerve and stapedius muscle have been reported [29]. Since the malformed tissues originate from the first and second branchial arches, with the facial nerve also being one of the nerves of those arches, retinoid-induced embryopathies of the middle ear are likely to be due to early effects of retinoic acid on the first and second arches [23]. Middle ear anomalies are consistent with the conductive hearing loss reported in patients with isotretinoin syndrome [30].

Defects of the external ear commonly ensue, both in animal models and in the developing human, following early exposure to retinoids [28,31,32,6], and are associated with anomalies of the first branchial arch [33]. The critical period for exposure appears to be two to five weeks postconception in the human, although this may not be clinically exact [32]. The deformities include anotia, aural atresia, microtic, low-placed and dorsally situated pinna, and narrowing of the external auditory canal [28,29]. Patients
with microtia and atresia are usually characterized by conductive hearing loss in the affected ear.

Exposure to retinoids at later developmental stages in the mouse leads to anomalies of the inner ear [18,20]. In utero exposure to high dose retinoic acid at E9 specifically leads to embryopathies of the cochlea, semicircular canals, utricle, saccule, endolymphatic duct, cochleovestibular ganglia and otic capsule. In extreme cases, the inner ear does not advance beyond a cystic-like structure that lacks distinct organization into pars superior and pars inferior portions (vestibular and cochlear portions respectively) (Figure 2). These inner ear anomalies are attributed to disruption in the inductive otic epithelial-periotic mesenchymal interactions that initiate at E9-E9.5 and guide development of these cochlear, vestibular and capsular structures [34]. Hindbrain segmentation and Krox-20 expression are not disturbed, suggesting that inner ear dysmorphogenesis by retinoic acid exposure at E9 is not a consequence of hindbrain abnormalities. Retinoic acid-induced inner ear embryopathies in the mouse bear resemblance to clinical findings in patients maternally exposed to isoretinoin during early gestation, in whom hypoplastic semicircular canals, abnormal endolymphatic sacs, utriculo-saccule deformities, and dysgenesis of the cochlea have been observed [29,35].

**How retinoids mediate their effects**

To gain insight into how retinoic acid targets inner ear development, it is important to understand the many pathways through which retinoids can exert their pleiotropic control. This control exists at many levels and is inclusive of retinoid binding proteins, retinoid receptors, and synthetic and metabolic enzymatic pathways.

**Cellular retinoid binding proteins**

The cellular retinoid binding proteins are a family of proteins that participate in
control of retinoid signaling and include the cellular retinol binding proteins (CRBP I and II) and the cellular retinoic acid binding proteins (CRABP I and II). Retinol, the circulating form of vitamin A, binds with high affinity to CRBP I and II whereas retinoic acid specifically binds to CRABP I and II [36,37,38]. CRABP and CRBP act as the major binding proteins for retinoic acid and retinol during development [39,40], and exhibit specific spatio-temporal expression patterns during otic morphogenesis. CRABP is apparent in the otic placode as early as E8, with high levels of transcripts present in the otic pit at E9 (Figure 3). CRBP signal is also evident in the epithelium of the E9 otic pit. By E10.5, CRABP I, II, and CRBP I are all present in the developing inner ear. CRABPII is expressed in the periotic mesenchyme but is not visible at this stage (E10.5) in the otocyst itself or the anlagen which derive from it [41,42], whereas CRBP I and CRABPII are found in the dorsal and ventral otocyst respectively [42]. Expression of CRBPI, CRABPI and CRABPII persists in sensory structures of the inner ear during subsequent stages of development.

Given the distinct expression patterns of CRBP and CRABP in the inner ear at critical stages of morphogenesis, one would anticipate an important function for these cytoplasmic proteins during development. Prior to the generation of null mutations of these binding proteins, several possible roles were hypothesized. These include a role for CRABP as a shuttle between the cell cytoplasm and nucleus to promote uptake of retinoic acid [43,44] and the sequestering of free retinoic acid to reduce the amount available for binding to retinoid receptors [45,46,47,48]. CRBPI was suggested to function in the generation of retinoic acid in cells requiring high levels for proper function and in demarcating regions of the inner ear sensory epithelia that possess regenerative capacity [49]. However, various combinations of single, double and triple null mutations of CRABP and CRBP provided no overt indication of inner ear anomaly or changes in auditory brainstem responses [36,50]. Retinoid binding proteins may thus not be
essential for proper development and function of the inner ear. Alternatively, conditions of vitamin A deficiency might be needed to unmask possible functions of CRBP in the inner ear since CRBPI null mice, when maintained on a vitamin A poor diet, develop a vitamin A deficiency syndrome [36]. Functional redundancy between CRBPI and recently identified CRBPIII and CRBPIV [51,52] may also come into play although expression patterns of CRBPIII and CRBPIV have not yet been defined in the inner ear [36].

**Retinoic acid synthesizing enzymes**

Enzymes that facilitate retinoic acid synthesis contribute to the maintenance of retinoic acid homeostasis. Neither embryonic nor adult animal cells produce retinoic acid de novo. Instead, dietary sources must provide provitamin A carotenoids and retinyl esters which are then converted into retinol [53]. Conversion of inactive retinol into active retinoic acid involves two oxidative reactions: in the first, retinol is oxidized to retinaldehyde by alcohol dehydrogenases, and in the second, retinaldehyde is efficiently oxidized into retinoic acid by retinaldehyde dehydrogenases (Raldh) [54].

Of the various isoforms of Raldhs thus far identified [54,55], four have been shown to be expressed in the developing inner ear (Raldh 1, 2, 3, 10). Importantly, their expression is confined to the otic epithelium, implicating the otic epithelium as a potential source of retinoic acid that may influence inner ear development. During early inner ear development (E9.5), expression of Raldh1 and Raldh2 is limited to the dorsal otocyst [36], with expression of Raldh2 being relatively weak. Subsequently (E10.5-E12.5), Raldh1 is detected in the developing endolymphatic duct, which arises from the dorsal otocyst (Figure 4), whereas Raldh2 becomes localized to a restricted lateral quadrant of the E10.5 otocyst [56]. At this developmental stage (E10.5), expression of Raldh3 is restricted to the region of the otocyst adjacent to the forming endolymphatic duct and the
ventral otocyst (Figure 4). Later in development (E14.5), Raldh1 is found in all three semicircular canals, the endolymphatic duct, and cristae, and Raldh2 to differentiating nonsensory regions of the cochlea and the vestibular region adjacent to the crista ampullaris. As development progresses, so do the expression domains of Raldh3, which now include the medial aspect of the otocyst, the endolymphatic duct, the lateral otocyst near the forming horizontal semicircular canal, and the ventral region of the forming cochlea. Expression of Raldh1 persists at E18.5 in the cristae and the newly developed maculae of the utricle and the saccule [36]. Raldh2 expression is limited to the vestibular portion of the inner ear, the stria vascularis, and Reissner’s membrane, whereas Raldh3 is more broadly expressed in the endolymphatic duct, the crista, the saccular and utricular sensory epithelia, the spiral ganglion, and the stria vascularis [36]. Recently identified Raldh10 is expressed in organs involved in endolymph homeostasis, being noted in the inner ear initially in the endolymphatic system and subsequently in the stria vascularis [57].

**Retinoic acid metabolizing enzymes**

The distribution and concentration of retinoic acid in the developing embryo is finely controlled by the complementary roles of retinoic acid synthesizing and metabolizing enzymes [58]. The Cyp26 group of enzymes (Cyp26A1, -B1, -C1) is part of the cytochrome P450 superfamily of enzymes, and they are specifically involved in catalyzing the conversion of retinoic acid into more polar hydroxylated and oxidized derivatives, a step in their metabolic breakdown [53,59,60]. A function of Cyp26s is to generate the precise pattern of retinoic acid present in the developing mouse embryo [53,61].

Cyp26A1 and Cyp26B1 are expressed at high levels during early otic development. At E9.5, expression of Cyp26A1 is observed in the caudal region of the
otocyst epithelium and in the axial mesenchyme just caudal and ventral to the otocyst. Cyp26B1 is present in the dorsal region of the E9.5 otocyst. By E10.5, expression of Cyp26A1 is strong in the ventral otocyst and mesenchyme surrounding the lateral and ventral otocyst wall, whereas only two focal regions of the lateral otocyst and prospective sensory epithelium continue to express Cyp26B1 (Figure 5). Expression of Cyp26A1 continues to be strong in the mesenchyme adjacent to the forming semicircular ducts and ventral portion of the E12.5 inner ear (Figure 5), but by E14.5 becomes restricted to the endolymphatic duct and cochlear epithelium. At this point (E14.5-E18.5), Cyp26B1 expression is limited to the sensory epithelium of the saccule, utricle, cristae, and cochlea. With the exception of the endolymphatic duct, Cyp26A1 is no longer evident in the E18.5 inner ear [36].

Cyp26C1 appears to play a more limited role in inner ear development. At E10.5, Cyp26C1 is present in the mesenchyme caudal to the E10.5 otocyst and in the ventral otocyst epithelium directly adjacent to the Cyp26C1-expressing mesenchyme [36,58]. Expression of Cyp26C1 continues in the cochlear epithelium of the E12.5 otocyst, however expression becomes negligible by E14.5.

**Retinoid receptors**

There are two classes of retinoid receptors, both of which are nuclear receptors and each of which has 3 subtypes: the retinoic acid receptors (RARs; α, β, γ) and the retinoid X receptors (RXR; α, β, γ). These nuclear receptors bind as either RXR homodimers or RAR/RXR heterodimers to retinoic acid response elements (RAREs) on target genes and function to facilitate transcription and gene expression [62,63]. Distinct, non-overlapping expression domains of all three RAR isoforms are present in the inner ear, beginning in the early otocyst stage and continuing into adulthood [64]. This diverse expression pattern of RARs and RXRs during embryonic development is thought to
delineate specific domains of retinoic acid action [56].

Specific isoforms of the retinoic acid receptors (RAR) have been detected as early as E10.5 in the developing inner ear (Figure 6). At this stage the β and γ isoforms of RAR are expressed in the periotic mesenchyme surrounding the anlagen of the developing endolymphatic duct. RARα is restricted to a small dorso-lateral area of the otic epithelium. By E12.5, all three RAR isoforms (α, β, γ) display distinct patterns of expression in either the otocyst or the periotic mesenchyme. In brief, RARα is evident throughout the otocyst epithelium, in the medial and lateral periotic mesenchyme, and in the dorsal aspect of the developing hindbrain. RARβ and RARγ expression persists in the condensing mesenchyme of the forming otic capsule [64], with RARβ also being expressed in the dorso-medial otocyst wall at the site of the presumptive vestibular epithelium. Expression of RARα persists in the cochlear and vestibular portions of the E14.5-E16.5 inner ear, although not as abundantly as previously, and in the organ of Corti until after birth. Between E14.5-E18.5, expression of RARγ remains primarily in the developing otic capsule and RARβ in tissues of mesenchymal origin.

The retinoid X receptors RXRα and RXRβ demonstrate a rather ubiquitous pattern of expression in the modiolus, cochlear sensory epithelium, spiral limbus, and stria vascularis of the E18.5 inner ear. The expression of RXRγ, by contrast, is much more restricted and is most visible in the spiral ganglion and Kolliker's organ [65]. RXR signal (α, β, γ) is not detected in the otic capsule at these developmental ages.

Based on the somewhat overlapping expression patterns of the various retinoid receptors in the developing inner ear, functional overlap between the different isoforms would seem a likely possibility. Consistent with this idea, RARα, RARβ, and RARγ null mutant mice did not reveal any morphological inner ear anomalies. This was also the case for RARα/RARβ and RARβ/RARγ double null mutant mice. In contrast, RARα/RARγ double null mutant mice demonstrated severe dysmorphogenesis of the
inner ear [64] that will be discussed in further detail in the retinoid deficiency section that follows.

**Changes in gene expression by retinoic acid excess**

Deviations from optimal concentrations of retinoic acid induce the ear phenotype by modifying the expression of target genes and signaling molecules critical to otic morphogenesis. An overview of key signaling molecules affected by changes in retinoic acid signaling at different stages of development is provided below.

*Goosecoid* gene expression is altered in the branchial region at E10.5 following maternal retinoic acid treatment at E8 plus 5 hours. This change in *goosecoid* correlates with specific teratogenic effects in the middle ear, most notably affecting the tympanic ring and middle ear ossicles [24]. Modified expression of *goosecoid* may be associated with an altered population of neural crest derived mesenchymal cells in the branchial arches [25]. Similarly, treatment of zebrafish embryos with retinoic acid leads to anomalies of craniofacial structures originating from neural crest cells in correlation with loss of *dlx* expression [66]. Recent studies suggest that retinoic acid teratogenicity derives from modified signaling from the pharyngeal (branchial) arch epithelium, leading to abnormal activation of genes in the cranial neural crest cells [23]. These epithelial signals include endothelin1 (*ET-1*) and *Fgf8*, which in turn induce *Dlx* gene expression in the incoming neural crest cells of the first arch. The malleus and incus, derived from the first and second branchial arches, and the stapes, a second arch derivative, are malformed or absent in mice with a targeted mutation of *ET-1* [67]. Retinoic acid-induced craniofacial anomalies are prevented when whole mouse embryo cultures are treated with folinic acid, which leads to significantly increased expression of *ET-1* and *dHAND* [68].

The molecular mechanisms underlying the action of retinoic acid on the inner ear are complex. Inner ear anomalies provoked by conditions of excess retinoic acid occur
in association with altered \textit{Dlx5} signaling of inductive tissue interactions. Downregulation
in \textit{Dlx5}, which participates in signaling from the otic epithelium to surrounding periotic
mesenchyme, ensues in the developing mouse inner ear in response to in utero exposure to high dose retinoic acid and in association with inner ear anomalies (Figure 7) [69]. This change in Dlx5 expression is not a direct response to retinoid signaling, but instead occurs indirectly as a consequence to retinoid-mediated changes in signaling by fibroblast growth factor 3 and -10 (\textit{Fgf3} and \textit{Fgf10}), which are essential to normal inner ear development. This makes sense given that sequence analysis of related \textit{Dlx3},\textit{4} reveals elements that resemble retinoic acid response elements (RAREs) but none that match exactly [70], while the 5’ flanking region of \textit{Xenopus Dlx2} does not contain sequences that match consensus RAREs [71]. On the other hand, sequence analysis of the \textit{Fgf3} inner ear enhancer demonstrates the presence of several different putative binding sites where retinoid signaling could directly control \textit{Fgf3} expression [72]. Potential retinoid binding sites have also been identified in a 4.5-kb fragment of the 5’ upstream region of \textit{Fgf10} [73]. Targeted deletion of \textit{Dlx5} and of \textit{Fgf3/Fgf10} leads to inner ear anomalies that resemble some of the anomalies produced by teratogenic levels of retinoic acid [74,75,76]. Furthermore, loss of \textit{Fgf3} and/or \textit{Fgf10} is correlated with downregulation in \textit{Dlx5} in the dorsal otic ectoderm, where it is exclusively expressed from E10.5 onward.

Of significance, \textit{Dlx5} is expressed in the epithelium of the developing otocyst, but not in the periotic mesenchyme from which the otic capsule forms. This finding indicates that anomalous otic capsule development, a consequence of targeted \textit{Dlx5} mutation and retinoid exposure, cannot be directly accounted for by loss of \textit{Dlx5}. Transforming growth factor-beta 1 (TGF\textit{\beta}_{1}), an essential contributor to otic capsule formation [77,78,79,80], is downregulated in the inner ear both by excess retinoic acid exposure [34] and loss-of-Dlx5-function (Figure 8) [69,81]. TGF\textit{\beta}_{1} may thus be one of the
downstream mediators through which Dlx5 elicits its otic capsule morphogenetic effects. In support of this, supplementation of cultured inner ear mesenchyme and epithelium with TGFβ1 rescues the cultures from the chondrogenic defects produced by Dlx5 deletion [69] or high dose retinoic acid treatment [34]. Besides TGFβ1, exposure to excess retinoic acid interferes with molecular signaling mechanisms downstream of the ligand [82]. When exposed in utero to high dose retinoic acid, malformations of the inner ear are produced that correlate with significant decreases in TGFβ receptor type II (TGFβRII) and Smad2, i.e. a downstream substrate of TGFβ receptor type I (TGFβRI).

Bone morphogenetic protein 4 (BMP4), a member of the TGFβ superfamily, participates in the epithelial-mesenchymal interactions that guide otic capsule chondrogenesis [80,83] and is subject to retinoic acid control. Repression of BMP4 expression, potentially facilitated by RARγ in the periotic mesenchyme [36,84], is evoked in cultured otocyst cells and in chicken otocysts in response to exogenous retinoic acid. BMP4 acts downstream of Dlx5 in semicircular canal formation [81]. Thus retinoid control of BMP4 may be indirectly facilitated by changes in Dlx5 expression. Alternatively, since RAREs have been identified in the promoter region of the BMP4 gene [85], this control may occur as a direct response to retinoic acid. In the zebrafish, BMP signaling activates foxi1 directly and dlx3b indirectly [86], factors which provide competence to form the inner ear [87,88]. Exposure to retinoic acid leads to misexpression of foxi1, but does not affect dlx3b expression [89].

**Retinoid deficiency and the inner ear**

Vitamin A deficient embryos lack an endogenous source of retinoids, and as a result, many developing organs requiring retinoic acid for development are malformed. Retinoid signaling is essential for normal development of the inner ear. In the vitamin A deficient quail, which lacks hindbrain rhombomeres 5-7 (r5 - r7), the otic vesicles are
typically positioned anterior to the first pair of somites, next to tissue with an r3 identity, with small ectopic vesicles just caudal to the primary otocyst [90]. These ectopic vesicles resemble the ectopic vesicles noted in the vitamin A deficient rat [11]. A reduction or absence of the vestibule-acoustic ganglion and absence of sensory organ development, assessed using markers of presumptive sensory patches, i.e. BMP4, Prox1 and Msx1, has been observed [90]. The ventral-most expression of Dlx5 is reduced and the ventral-lateral otocyst marker SOHo-1 absent in E2.5 vitamin A deficient embryos, while at E3.5 Pax2 and EphA4 are diminished and ectopically expressed in ventral and dorso-lateral regions of the otocyst respectively.

Offspring of rats maintained on a vitamin A deficient diet (50 µg/all-trans retinoic acid/g) exhibit malformed otic vesicles, whereas embryos from dams fed 25 µg/all-trans retinoic acid/g or less show small immature otic vesicles, one located in the normal location and a second more caudal vesicle [11]. In some cases, where rats are fed only 0.5 µg/all-trans retinoic acid/g, embryos show absence of otic vesicle formation. As the level of retinoic acid is reduced, a loss of caudal hindbrain segmentation is observed and the incidence of otic anomalies increases [11]. Thus otic anomalies may ensue as a secondary consequence of hindbrain abnormalities.

In the mouse, retinoic acid deficiency due to double null mutation of RARα and RARγ produces inner ears with poorly developed cochleae, absence of semicircular canals, disorganized sensory epithelia and a malformed otic capsule [64]. Analysis of Raldh2 null mutant embryos at earlier developmental stages due to embryonic lethality shows hypoplastic otocysts that are abnormally distant from the hindbrain neuroepithelium [14]. This neuroepithelium (i.e. Raldh2 null) is characterized by only weak expression of Fgf3, suggesting diminished FGF in the hindbrain as a potential indirect cause of the poor otocyst development. However, between E9-E10, FGF3 and FGF10 become expressed in the developing otic cup and vesicle [73,91,92]. Treatment
of otic explants with pharmacological retinoid signaling inhibitors at E10.5, a timepoint by which the otocyst can develop in vitro even in the absence of the influence of the rhombencephalon, also show immature otic vesicles in association with diminished levels of FGF3 and FGF10 expression (Figure 9). Thus retinoid embryopathy of the inner ear may also occur independent of an affect of the hindbrain. In the zebrafish, loss of retinoic acid signaling leads to reduced otic vesicles with only one small otolith and impaired protrusions of the semicircular canals [89]. This diminution in endogenous retinoic acid, produced by treatment of embryos with a pharmacological inhibitor (4-diethylamino-benzaldehyde), interferes with fgf3 signaling, leading to a significant reduction in its expression in comparison to control embryos [89].

Conclusion

Retinoic acid is essential to otic morphogenesis, with finely controlled regulatory mechanisms in place at many levels. However, when changes in retinoid signaling occur due to deviations in retinoid exposure and/or perturbation in control factors, substantive inner ear defects ensue. Given the myriad of congenital malformations produced by retinoid deficiency and the increased risk for malformations following excess retinoid exposure, identification of the molecular pathways and regulatory networks that underlie retinoid embryopathies remains an important challenge for the future.

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References Cited


Figure Legends

**Figure 1.** RA treatment of mouse embryos at E8.5 leads to loss of the tympanic ring and middle ear ossicles. Bone and cartilage staining of otic specimens from E17.5 (A) control and (B) RA-treated embryos shows a partial loss of the tympanic ring (tr) and absence of the malleus (m), incus (i) and stapes (s) in the middle ear of an RA-treated embryo in comparison to that of a control embryo. Arrowheads in (B) indicate cartilaginous condensations reminiscent of these structures. The styloid cartilage (sy), gonial bone (g) and squamous bone (sq) were also affected. MC, Meckel's cartilage. With permission requested from Zhu et al., *Biochemical and Biophysical Res. Comm* 235, p750 (1997).

**Figure 2.** RA treatment of mouse embryos at E9 leads to anomalies of the inner ear. (A) Control inner ear showing normal formation of a coiled cochlear duct (C), semicircular duct (D) and vestibule. The region of the developing utricle (U) is indicated. This is contrasted by the in utero retinoic acid-exposed inner ear which demonstrates (B) cystic outgrowth of the cochlear duct (C) and a common utriculo-saccular area (U-S). The developing semicircular ducts are indicated (D). In (C), an extreme case of embryopathy is shown, in which the inner ear develops as only a cystic structure. Reprinted with permission from John Wiley & Sons, Inc. (*Teratology* Vol. 53, No. 5, 1996, p. 298, Frenz et al.).

**Figure 3.** Cellular retinoid binding proteins are expressed in the developing inner ear. Parasagittal sections of an E9 mouse embryo, hybridized for CRABP and CRBP. Expression of CRABP and CRBP is evident in the otic cup (arrows). Reproduced with permission of the Company of Biologists (*Development* Vol. 111, No. 1, 1991, p. 57,
Ruberte et al.).

**Figure 4.** Retinoic acid synthesizing enzymes are expressed in the developing inner ear. Whole mount in situ hybridization showing expression of (A) Raldh1, (B) Raldh2, and (C) Raldh3 in the E9.5 mouse inner ear (arrows). Schematic summary (right) of the gene expression pattern for Raldh1, Raldh2, and Raldh3 (in red). Ed, endolympathic duct; G, anlagen of the stato-acoustic ganglion. Yellow demarcates otic epithelium; blue, periotic mesenchyme. Reprinted with permission from John Wiley & Sons, Inc. (*J. Neurobiol.* Vol. 66, No. 7, 2006, p. 693 and 694, Romand et al.).

**Figure 5.** Retinoic acid catabolizing enzymes are expressed in the developing inner ear. Serial section in situ hybridization demonstrates the expression of Cyp26A1 in the (A) ventro-lateral otic epithelium and adjacent mesenchyme at E10.5 and in the (C) developing cochlea opposite the prospective sensory epithelium (arrow) and periotic mesenchyme adjacent to the forming horizontal semicircular canal (small arrow) at E12.5. Weak signal is detected in the mesenchyme adjacent to the cochlea (large arrow). Cyp26B1 transcripts are limited to two small regions in the lateral and ventromedial otocyst (arrows) at E10.5 (B) and to mesenchymal tissue surrounding the horizontal semicircular canal and cochlea (arrows) at E12.5 (D). Schematic to the right summarizes this expression data in red. Green indicates otic epithelium; light blue, periotic mesenchyme. Reprinted with permission from John Wiley & Sons, Inc. (*J. Comp. Neurol.* Vol. 46, No. 5, 2006, p. 649 and 650, Romand et al.).

**Figure 6.** Retinoic acid nuclear receptors are expressed in the developing inner ear. Serial section in situ hybridization showing the pattern of RARα, RARβ and RARγ expression in the E10.5 and E12.5 mouse inner ear (Ot). In brief, RARα is expressed in
a limited dorso-lateral area of the otic epithelium at E10.5 (A), but becomes more extensively present in these regions at E12.5 (D). Expression is also noted at E12.5 in the medial and lateral mesenchyme. B,C,E,F) RARβ and RARγ are mainly expressed in the periotic mesenchyme, although RARβ is noted in a restricted region of the dorso-medial otic epithelium (E). Hb, hindbrain; Ed, endolymphatic duct anlagen; Cc, cochlear canal; Vc, vestibular canal. A “summary” of this expression data is depicted at the right. Reprinted with permission from John Wiley & Sons, Inc. (J Neurobiol 66, No. 7, 2006, p. 694, Romand et al.). Permission requested from Romand et al., Mech Devel, p. 214 (2002).

**Figure 7.** In utero exposure to retinoic acid alters Dlx5 expression. Whole mount in situ hybridization for Dlx5 in control and retinoic acid-exposed mouse embryos. A) E9.5, showing comparable levels of Dlx5 expression in control and all-trans retinoic acid (atRA) exposed otocysts (arrows). B) E10.5, showing diminished Dlx5 in the atRA-exposed otocyst compared with the control otocyst (arrows). Magnified view of each otocyst is provided below the respective embryo. Reprinted with permission from John Wiley & Sons, Inc. (Birth Defects Res (Part B) Reprod.Toxicol, Vol. 83, No. 2, 2008, p. 139, Liu et al.).

**Figure 8.** Expression of TGFβ₁ is diminished by in utero exposure to retinoic acid and by targeted Dlx5 mutation. A,B) E12 mouse inner ears, showing dense reaction product for TGFβ₁ in the condensing mesenchyme, particularly opposite the presumptive horizontal semicircular duct, of the control inner ear contrasted by only a pale immunostain in the periotic mesenchyme surrounding the malformed retinoic acid-exposed inner ear (B). C,D) E14 Dlx5 mutant inner ears, showing immunostaining for TGFβ₁ in the chondrifying otic capsule (dorsal region pictured) of a wildtype specimen.
(C). However, in the malformed Dlx5 null inner ear (D), the otic capsule mesenchyme has not yet condensed, and immunostaining for TGFβ1 is not evident. Reprinted with permission from John Wiley & Sons, Inc. (Teratology, Vol. 61, No. 4, 2000, p. 300, Frenz and Liu; Birth Defects Res (Part B) Reprod.Toxicol, Vol. 83, No. 2, 2008, p. 139, Liu et al.).

**Figure 9.** In vitro treatment with a pharmacological inhibitor of retinoid signaling affects otocyst development and FGF expression. A, B, C) Organotypic cultures of the inner ear, in which retinoic acid deficiency was modeled by treatment with the aldehyde dehydrogenase inhibitor citral (72 hours in vitro). A) Control otocyst (demarcated by arrows), showing outpocketing of the developing endolymphatic duct and sac anlagen (ED) and the forming cochlear duct (CD). B, C) Citral-treated otocysts (200 µM and 500 µM respectively), showing a more marked extent of perturbation in otocyst development with increasing concentration of citral. D) RT-PCR analysis of FGF10 in control and citral-treated organotypic cultures. lane 1, Control; lane 2, citral, 200 µM; lane 3, citral, 300 µM; 48 hrs in vitro. FGF10 is downregulated by citral, with more marked diminution in FGF10 evident with increasing concentration of the reagent. E) β-actin control, showing comparable bands in lane 1 (control), lane 2 (citral, 200 µM) and lane 3 (citral, 300 µM); 48 hours in vitro.
Fig 6

Fig 7