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Can you hear me now? Understanding vertebrate middle ear development

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1. ABSTRACT

The middle ear is a composite organ formed from all three germ layers and the neural crest. It provides the link between the outside world and the inner ear, where sound is transduced and routed to the brain for processing. Extensive classical and modern studies have described the complex morphology and origin of the middle ear. Non-mammalian vertebrates have a single ossicle, the columella. Mammals have three functionally equivalent ossicles, designated the malleus, incus and stapes. In this review, I focus on the role of genes known to function in the middle ear. Genetic studies are beginning to unravel the induction and patterning of the multiple middle ear elements including the tympanum, skeletal elements, the air-filled cavity, and the insertion point into the inner ear oval window. Future studies that elucidate the integrated spatio-temporal signaling mechanisms required to pattern the middle ear organ system are needed. The longer-term translational benefits of understanding normal and abnormal ear development will have a direct impact on human health outcomes.

2. INTRODUCTION

The function of the middle ear is to transmit and amplify sound from the external environment, across the middle ear cavity, to the inner ear (1, 2). The middle ear is a composite organ, produced from all three germ layers, and the neural crest contributing to its development (3-7). Studies of the morphology, spatio-temporal development and molecular genetic interactions between the various elements reveal a complex set of coordinated interactions that researchers are still trying to unravel.

Producing a functional link between the outside world and perception of the environment is crucial to human survival. Abnormal external and middle ear development leading to congenital conductive hearing loss affects about 10% of the 1 in 300-500 newborns with moderate to severe hearing loss. The number of affected individuals increases with age, due to factors such as stapes fixation (otosclerosis), infections such as otitis media, tinnitus, tumors, perforated eardrums, temporal bone fractures, cholesteatoma and other traumas. An

understanding of developmental mechanisms required to pattern the middle ear will contribute to improved therapeutic approaches to hearing defects.

Body tissue reflects the majority of sound waves, so in order to hear, animals relay sound via the middle ear, an air-filled cavity. In mammals, sound vibrates the tympanum, and relays sound via the three chain ossicles (malleus, incus and stapes), to the basilar membrane underlying the footplate, which is inserted into the oval window. In non-mammalian gnathostomes, a single endochondral bone, the stapes/columella performs the same function. The columella is the developmental equivalent of the mammalian second pharyngeal arch component, the stapes (8). The piston like movement of the stapes footplate in the oval window transduces airborne sound waves from the environment to the endolymph of the inner ear. Signal amplification is achieved by the lever action of the ossicular chain, and an increase in the magnitude of the signal. The tympanic membrane covers a large surface area compared to the oval window. The reduction in area increases the pressure at the footplate and thus increases the amplitude of the signal. The footplate is held in place by the annular ligament, which allows the stapes/columella to move.

The focus of this review is to describe the current knowledge of the role of signaling pathways and associated factors in middle ear development. Numerous signaling mechanisms, including the *Hoxa2* selector gene, Retinoic acid (RA), Fibroblast Growth Factor (FGF), Bone Morphogenetic Protein (BMP) and WNT family members and are required for proper development. To aid understanding of the signaling and genetic mechanisms, I will briefly introduce and review amphibian, reptile, avian (chick), and mammalian (mouse) middle ear morphology. Detailed morphological descriptions of these models have been extensively reviewed elsewhere (9-13). Primarily using studies of chick and mouse, the origin of the neural crest contribution and signaling mechanisms that patterning this cell population will be discussed. This will be followed by discussion of the genes known to be involved in mesenchymal condensation, chondrogenesis and osteogenesis, and patterning of the endochondral and intramembranous skeletal elements. Lastly, signaling during formation of the tympanic membrane, external auditory meatus, middle ear cavity, and Eustachian tube formation, together with muscles, nerves, and joint formation will be discussed.

Genetic, functional and morphological studies are enhancing our understanding of middle ear induction, migration, patterning and endochondral ossification, but a comprehensive model is not yet available. Studies involving disruption of candidate genes, and mutant mice with middle ear phenotypes (Table 1), point to the large number of molecular players necessary for development of this composite organ. Due to space limitations, middle ear evolution (14, 15) and translational research will not be covered here.

3. MORPHOLOGY

In this section, I will provide a brief description of amphibian, reptile, avian and mammalian middle ear morphology. This section is to aid in the understanding of the discussion on signaling pathways and other molecules to follow. A large body of published work is available for detailed information on morphology (8, 9, 11-13, 15).

3.1. Amphibian morphology

The otic apparatus has five cartilages that form the middle and external ear. From distal to proximal these are: the tympanic annulus; extrastapes (pars externa plectra); stapes (pars media plectra); stapedial footplate (pars interna plectra); and the operculum (origin unknown). The otic apparatus can form from single or multiple cartilaginous condensations in anurans and has diverse forms among the various species (Figure 1A, B; frog, terrestrial salamander *Ambystoma*). *Xenopus laevis*, for and example, has two condensations, namely the pars externa plectra; and, the pars interna and media plectra.

There is extensive classical literature on the amphibian middle and external ear [reviewed in (12, 13, 16)]. Many studies have focused on tracing neural crest cells over extended time frames, from the larval stages through metamorphosis to adulthood. However, there is no cartilage in the larva, and cartilage arises de novo at metamorphosis. Modern fate maps using fluorescent dye labeling methods (17), and novel transgenic methods (18) have revolutionized the available fate maps.

Three neural crest streams contribute to cartilages in the middle and external ear (Figure 1C) (19). The mandibular stream neural crest forms the tympanic annulus (external ear) supporting the ring-like cartilage surrounding and supporting the drum-like tympanic membrane. The hyoid stream neural crest gives rise to the pars externa plectri (extrastapes) that articulates with the tympanum distally, and pars media plectri (stapes), proximally. Finally, and unexpectedly, data from ROSA26:GFP transgenic embryos showed that the branchial neural crest stream forms the pars interna plectri (stapedial footplate) that inserts into the oval window (18). The term branchial is generally used when referring to vertebrates with gills, whereas pharyngeal refers to the arches of higher vertebrates. The anterior and posterior domains of the otic capsule also arise from the branchial stream as determined by the mosaic labeled cells in the otic capsule and incompletely ossified prootic and exoccipital bones (18). These data provide evidence for a model where the stapes is a composite structure from numerous subpopulations of cells. These modern studies, of labeled neural crest derivatives, therefore support a model in which the stapes is a second/hyoid arch component, and the tympanic annulus is of mandibular/first arch origin (16, 18).

3.2. Reptile morphology

The function of the middle ear is similar between avian and reptile groups, although there are multiple structural forms within the reptilian lineage (Figure 1 D, lizard) (11, 20, 21). Among the reptiles, the tympanic membrane can be deep with an external auditory meatus; superficial to the skin requiring no external auditory meatus; or, even entirely missing. The tympanic membrane

Table 1. Genes reported to have middle ear phenotypes

Symbol, Name	Chromosome	Evidence	Ref(s)
Ap2, adipocyte protein KO	3 (13.9)	Transgenic, recessive	(106, 107)
Apaf1, apoptotic protease activating factor 1 KO	10	Transgenic, recessive	(108)
Bmp5, short ear (se)	9 (42)	Spontaneous, radiation-induced, recessive	(109)
Brn4 (Pou3f4), sex-linked fidget KO	X (48.4)	Transgenic, radiation-induced, semi-dominant	(88, 110, 111)
Cola1, collagen type 1alpha1 KO	11 (56)	Transgenic, insertional mutation, dominant	(112-114)
Crtl1, cartilage link protein 1 KO	13 (44)	Transgenic, recessive	(115)
Dlx1, distal-less homeobox 1 KO	2 (44)	Transgenic, recessive, epistatic	(72)
Dlx2, distal-less homeobox 2 KO	2 (44)	Transgenic, recessive, epistatic	(72)
Dlx5, distal-less homeobox 5 KO	6 (2)	Transgenic, recessive	(116, 117)
Edn1, endothelin 1	13 (26)	Transgenic, semi-dominant	(85)
Ednra, endothelin receptor type A KO	-	Transgenic, recessive	(87)
Ece1, endothelin converting enzyme 1 KO	-	Transgenic, recessive	(118)
Eya1, eyes absent 1 homolog KO	1 (10)	Inferred from Mutant Phenotype	(119)
Gas1, growth arrest specific 1	13	Inferred from Mutant Phenotype	(120)
Gsc, goosecoid KO	12 (52)	Transgenic, recessive	(100, 101, 121)
Hoxa1, homeobox A1	6 (26.3)	Trangenic, recessive, epistatic	(54, 122-125)
Hoxa2, homeobox A2	6 (26.3)	Trangenic, recessive	(61, 63, 64, 98)
Hoxb2, homeobox B2 KO	11 (56)	Transgenic, recessive	(126)
Hspy, hush puppy	8	Inferred from Mutant Phenotype	(127)
Insig1, insulin induced gene 1	5	Inferred from genetic interaction	(128)
Insig2, insulin induced gene 2	1	Inferred from genetic interaction	(128)
Msx1, homeobox msh-like 1 KO (Hox7)	13 (32)	Transgenic, dominant	(129)
Msx2, homeobox msh-like 2 KO (Hox8)	5 (21)	Transgenic, recessive	(84)
Myc, myelocytomatosis oncogene	15	Inferred from Mutant Phenotype	(130)
Bapx1/Nkx3-2, NK3 homeobox 2	5	Inferred from Mutant Phenotype	(29)
Noggin overexpressing transgenic	-	Transgenic, semi-dominant?	(131)
Osr1, odd-skipped related 1 KO	12	IGI	(132)
Osr2, odd-skipped related 2 KO	15	Inferred from Mutant Phenotype	(132)
Otx2, orthodenticle homog 2 KO	14 (19)	Transgenic, semi-dominant, epistatic	(133-135)
P73 KO	-	Transgenic, recessive	(136)
Pax9 KO	12 (26)	Transgenic, recessive	(137)
Prkra, protein kinase, interferon inducible double stranded RNA dependent activator	2	Inferred from Mutant Phenotype	(138)
Prx1, paired related homeobox 1 (MHox)	1	Transgenic, recessive, epistatic	(74, 139)
Prx2, paired related homeobox 2	2 (19)	Inferred from genetic interaction recessive, epistatic	(139)
Retinoic acid receptor alpha KO	11 (57.8)	Transgenic, recessive, epistatic	(140-142)
Retinoic acid receptor beta KO	14	Transgenic, recessive, epistatic	(140-142)
Retinoic acid receptor gamma KO	15 (57.4)	Transgenic, recessive, epistatic	(142)
Six2, sine oculis-related homeobox 2 homolog (Drosophila)	17	Inferred from genetic interaction	(68)
Tbx1, T-box 1	16	Inferred from Mutant Phenotype	(53, 143)
Tshz1, teashirt zinc finger family member 1	18	Inferred from Mutant Phenotype	(144)

can be covered and protected by flaps, scaly skin, or closed by muscle. The Eustachian tube can be wide open to the mouth, have a very small, short tube in the mouth, and in many cases connects both ears directly through the skull. In the case of crocodiles this tube runs dorsally over the brain, whereas in avians the Eustachian tubes join medially under the brain and then open into the mouth via a single outlet. This configuration allows for both pressure equalization and sound localization using the delay in reception between the two sides of the head.

3.3. Avian morphology

The columella is unique in that it develops in isolation from other endochondral bones (Figure 1E and 2). It resides in an air-filled cavity, joined to the pharynx by the Eustachian tube. The columella hangs in space, except for two contact points: the proximal stapedial footplate inserted into the oval window; and, the distal extracolumella arms inserted into the tympanic membrane. Although non-weight bearing, the bone is under tension between its contact points, pushing the tympanum outward into the external auditory meatus, creating a drum-like tension. As sound vibrations stimulate the membrane, they are transmitted to the columella, which bends and rocks the footplate in the oval window leading to movement of the endolymph and mechanotransduction by hair cells and finally to the brain, via the vestibulocochlear nerve (CNVIII) (22-24).

The proximal columella shaft and footplate are ossified, whereas the distal portion remains cartilaginous throughout life, and is required for flexion of the extracolumella arms inserted into the tympanic membrane. Ossification begins in the shaft at the proximal end, then in the footplate, and proceeds up the shaft toward the distal extracolumella, forming the joint region with the cartilaginous extracolumella (2, 25-27).

3.4. Mammalian morphology

The mammalian middle ear is comprised of an external, middle and inner ear for hearing (Figure 1F and 3). The external auditory meatus and pinna direct sound to the tympanic membrane, which flexes inward, toward the middle ear cavity. To form the auditory meatus, the first pharyngeal cleft surface ectoderm invaginates, and together with opposing pharyngeal pouch endoderm, sandwiches an

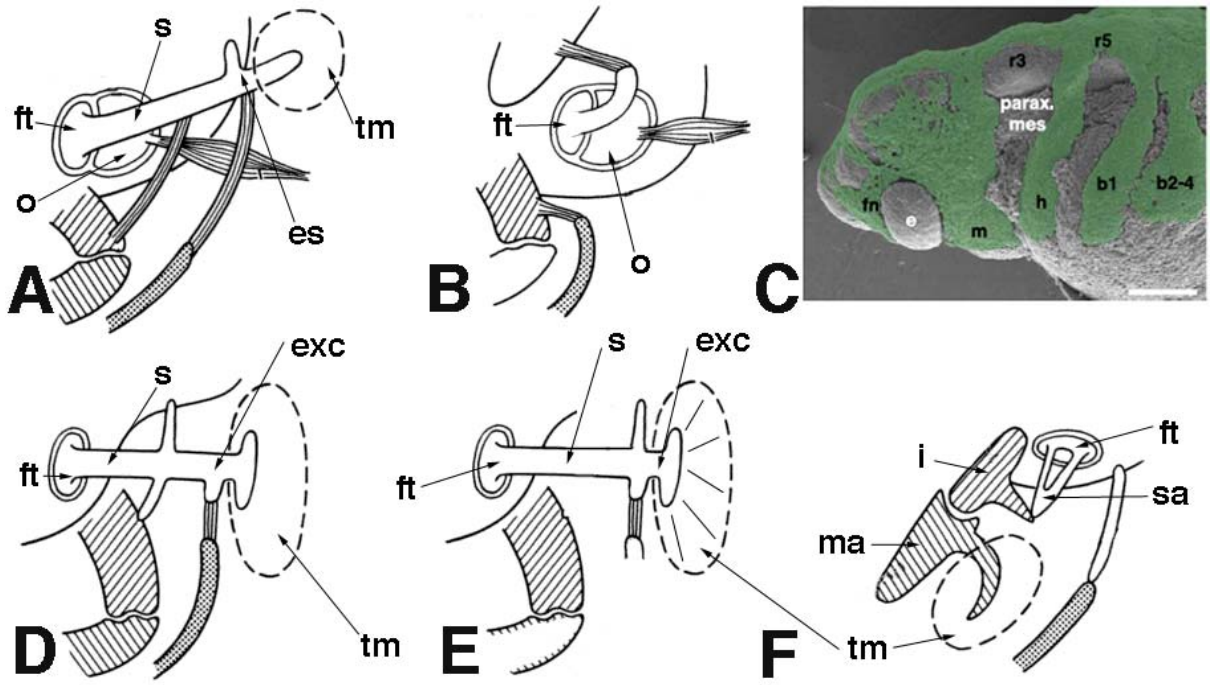

Figure 1. Variable forms of vertebrate middle ears. Stylized line drawings of (A) amphibian/frog, (B) amphibian/salamander (*Ambystoma*), (D) reptile/lizard, (E) avian and (F) mammal middle ear elements. (C) Neural crest streams (green) in the Mexican axolotl at late migration stages (stage 29/30). The manibular stream has reached its ventral-most position, and the hyoid and branchial streams have completed migration, but do not travel as far ventrally. Scale bar 200 μm. Abbreviations: b1-4, branchial arch streams; es, extrastapes; exc, extracolumella; fn, frontonasal neural crest; ft, footplate; h, hyoid stream; i, incus; m, mandibular crest; ma, malleus; o, operculum; parax. mes, paraxial mesoderm; r, rhombomere; s, stapes; sa, stapedial arch; ta, tympanic annulus; tm, tympanic membrane. Images reprinted and adapted with permission.

elastic fibrous mesenchymal-derived layer, forming the three-layered tympanum (28). The medial air-filled middle ear cavity houses the suspended three-ossicle chain (malleus, incus and stapes), with the tympanic ring and gonium distally. The tympanic ring supports the membrane, with the gonium anchoring it to the skull (29). Sound waves are amplified and transmitted between the tympanum and proximal inner ear oval window, via the articulated ossicular chain. The malleal manubrium process is inserted into the tympanum at the distal end of the chain, linked by the incudo-malleal joint (a v-shaped articulation between the wedged incus and malleal notch) to the incus, which acts as a bridge to the stapes (30, 31). The incus and stapes interact via the incudo-stapedial joint (a soft connection, semi-independent vibrating system), with the stapedial footplate inserting into the oval window proximally. The lever action of the three-ossicle chain transmits and amplifies sound to the stapes footplate, which acts like a piston to transduce the energy to the fluid-filled organ of Corti, where mechanotransduction occurs via hair cells to the auditory nerve.

All of the skeletal elements are neural crest derived; with the stapes being of second arch origin. The others are first arch derivatives. The three ossicles are endochondral bones, whereas the tympanic ring and gonium are dermal bones (29).

4. NEURAL CREST ORIGIN OF THE SKELETAL ELEMENTS

Neural crest cells are sometimes referred to as the fourth germ layer, as their derivatives are so numerous and diverse (32). Evidence for the neural crest origin of the middle ear skeletal elements comes from multiple studies in amphibian, chick and mouse (32). In amphibian, using dye and transgenic labeling studies (16-18, 33), in chick using the quail-chick chimera system (3, 34-37), and in mouse using transgenic r4 labeling (7).

Three streams of neural crest (mandibular, hyoid and branchial/pharyngeal) cells migrate from the dorsal neural tube rhombomeres to more ventral positions in the arches (Figure 1C and 4). These cells constitute the cranial neural crest population, also known as the ectomesenchyme. The mandibular stream originates from the posterior midbrain, rhombomere (r)1 and r2, giving rise to the upper and lower jaws from the first arch (Figure 4). The hyoid stream migrates from r4 to the second arch, giving rise to the hyoid bones and stapes/columella in all animals with this structure. The more caudal branchial streams originating from r6/7 give rise to the gills in amphibians and fish. In higher vertebrates, the hyobranchials, basihyoid and thyroid cartilages all derive from these more posterior pharyngeal arches (3, 34, 38,

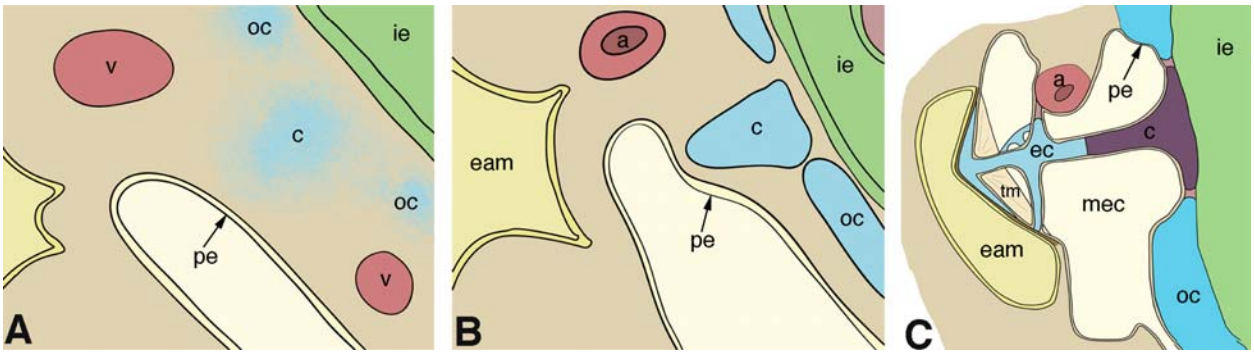

Figure 2. Schematic of columella development in the chick. Transverse orientation through the head at the level of the second pharyngeal arch; day 6 to14. (A) Ectomesenchyme cells condensing to form the columella and otic capsule, no chondrogenesis yet. (B) The pharyngeal endoderm and ectoderm of the external auditory meatus gradually move in apposition, with overt condensation (blue) and footplate insertion into the oval window occurring. The middle ear cavity is enlarging. (C) Ossification of the columella shaft and footplate has occurred (purple). The three arms of the cartilaginous extracolumella are in position with the extracolumella process pushing the tympanic membrane into the external auditory process. The middle ear cavity is connected to the pharynx via the Eustachian tube. Abbreviations: a, artery; c, columella; eam, external auditory meatus; ec, extracolumella; ie, inner ear; mec, middle ear cavity; oc, otic capsule, pe, pharyngeal endoderm; tm, tympanic membrane; v, blood vessels.

39). Crest from r3 and r5 contributes minimally to the hyoid stream, mostly undergoing apoptosis (40).

Unique to mammals, first arch neural crest derivatives include the malleus and incus that undergo endochondral ossification, whereas the tympanic ring and gonial bones that support the tympanic membrane are dermal/membranous bones (29). As with all vertebrates, the stapes undergoes endochondral ossification and is a second arch derivative (35).

Extensive transplant studies in the chick have provided a wealth of information on the skeletal origin of the columella. In early studies, mesencephalic and rhombencephalic neural crest grafts gave rise to hyoid and mandibular skeleton, including the otic region (5). Basal plate cartilages were only formed from chick cells irrespective of the level of transplant, and must therefore be of mesoderm origin. Grafts of anterior rhombomeres and mesencephalon resulted in labeling of the otic region, including a region of mixed quail/chick origin in the ventral otic capsule. In this study, the columella and squamosal were reportedly derived solely from neural crest. Later quail-chick chimeras and fine fate mapping studies showed a more restricted contribution, from the r4 region (with minor r3/5 contributions), sufficient to form the columella (3, 35, 41, 42). Noden's data argues for a mesoderm component forming a composite columella. Kontges and coworkers also failed to find quail neural crest within the footplate, postulating that the footplate may be of mesoderm origin, in agreement with Noden, although they did not formally test this possibility. Recall that in amphibians the branchial neural crest stream gives rise to the footplate (18). Thus, some uncertainty remains regarding the origin of the footplate in the chick columella with regard to a potential dual origin, which we are in the process of investigating.

Genetic labeling of second arch ectomesenchyme in double heterozygous hoxb1-cre mice revealed that r4 neural crest contributes to the lateral process of the malleus (similar to the proximal retroarticular process in the chick articular bone), cartilaginous otic capsule (identical to chick), and stapes (7). Other contributions to the middle ear were not labeled by this technique, and thus could not be assessed.

Having established that the skeletal elements of the middle ear are derived from the cranial neural crest, the genes that pattern the neural crest cells are the next point of discussion.

5. SIGNALING MECHANISMS PATTERN NEURAL CREST CONTRIBUTIONS

The neural crest, head mesoderm and adjacent epithelia all provide signals for development of the multiple skeletal elements. Formation of the pharyngeal arches, together with correctly targeted migration of the neural crest streams is critical to proper positioning of skeletal elements of the middle ear (43-46).

Retinoic acid (RA) plays a key role in the positioning of the middle ear. In Vitamin A-deficient (VAD) quail embryos the hindbrain is re-patterned, with r4 displaced caudally, adjacent to somites 1-5, and r5-7 failing to form at all (47). Inner ear placode formation and morphogenesis proceed normally, as induction relies on mesoderm signals until the 5-somite stage. Early gene expression of Fgf3, Fgf19 and Pax1, for example, are maintained in the mesoderm adjacent to the otic placode. Although rhombomere positioning is affected, the second pouch forms in approximately the right position, with compromised development and loss of Fgf8 expression in the pharyngeal endoderm and reduced ectodermal expression (48). The fate of r4 neural crest in these embryos has not been assessed, and VAD embryos only survive to embryonic day 4, so analysis of middle ear skeletal elements was not possible.

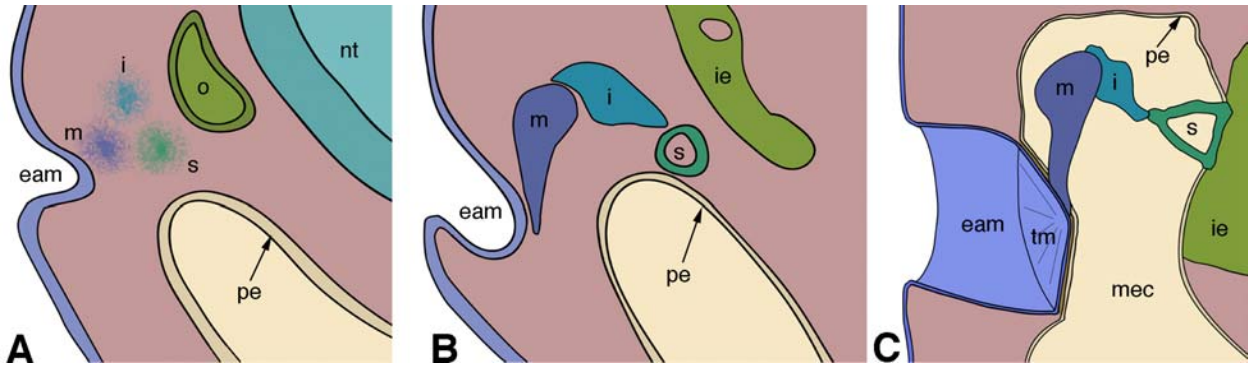

Figure 3. Schematic of mouse middle ear development. Schematic shown to include all three condensations; malleus (purple), incus (blue), stapes (green); E11.5 to adult. (A) The external auditory meatus is invaginating, with three condensations of the chain ossicles between the ectoderm and otocyst. (B) Malleal manubrium in contact with the ectoderm of the future tympanic membrane, articulating with the incus and stapes. (C) Tympanum is convex, with fully articulated ossicles and footplate inserted into the oval window. Middle ear cavity is air filled. Abbreviations: eam, external auditory meatus; ie, inner ear; i, incus; m, malleus; mec, middle ear cavity; nt, neural tube; o, otocyst; pe, pharyngeal endoderm; s, stapes; tm, tympanic membrane.

The r4 neural crest appears to maintain its identity when RA-soaked beads are implanted in the hindbrain, but displays aberrant migration into the first arch, resulting in formation of the retroarticular process cartilage ectopically in the first arch and loss of the columella. Mesenchymal expression of Hoxa2 extends anteriorly, altering signaling in the normally Hox-negative first arch, resulting in a partial gain of function, and transformation of first arch cartilage into second arch derivatives. The columella was missing in all treated embryos. Thus, the identity of neural crest cells is not affected by application of RA per se; rather, abnormal migration together with ectopic Hoxa2 in the first arch alters the local signals encountered by ectomesenchyme entering the arch (49).

Fgfr1 is required in arch mesoderm, with mouse mutants having compromised r4 migration, remarkably similar to *Tbx1*-/- mutant embryos (50). *Fgf8* and *Fgf10* are both downregulated in *Tbx1* mutants. While *Fgf8* is not involved in neural crest migration, it is required for patterning and crest cell survival post-migration. Loss of *Fgf10* alone produces no middle ear phenotype, suggesting redundant patterning mechanisms operate when it is lost, but no such redundancy exists for *Fgf8* (51, 52).

A *Tbx1* mutation affects multiple components of the hearing apparatus. In mutants, the second and more posterior arches do not form, affecting morphogenesis and patterning over a large region, resulting in aberrant skeletal development. With regard to middle ear elements, there is concomitant loss of the stapes, reduction of the first arch-derived incus to a cartilaginous nodule, and the malleus is noticeably hypoplastic, but all of the malleal elements are present. The tympanic membrane appears only mildly affected, with a shorter, thicker tympanic ring, forming a functional eardrum, with an inserted malleal manubrium. This suggests that interactions between the distal ectoderm, pharyngeal endoderm, and neural crest forming the tympanic ring are less affected in *Tbx1* mutants. The r4 neural crest migratory stream is present, but is misdirected into the first arch, resulting in *Hoxa2* expressing cells in the first arch, phenocopying *Hoxa2* expression mutants (see below). However, neural crest cells do not express *Tbx1*, only the mesodermal core of the arches express *Tbx1*, suggesting a non-cell autonomous effect, indirectly affecting the migratory pathway of the neural crest. *Crapb1*- and *Hoxa2*-positive neural crest cells in *Tbx1* mutants were analyzed by *in situ* hybridization. In these mutants neural crest cells migrated into the first arch, suggesting that the hindbrain mesoderm lacked the ability to restrict migration to the normal stream pathway (53).

In *Hoxa1* null mutants the tympanic ring is displaced rostrally, with the stapes fused to the otic capsule, and the malleus can appear hypoplastic. All other elements, including tensor tympani and stapedius muscles are normal. *Hoxa1*^{null}/*Hoxb1*^{RARE} double homozygote mice are missing all skeletal elements, except the incus, and have a strongly hypoplastic malleus and underdeveloped muscles. Hoxa1^{null}/Hoxb1^{null} double homozygotes are similar to *Hoxa1*^{null}/*Hoxb1*^{RARE} double homozygotes, but not all elements were analyzed. Reduced neural crest migration into the second arch was determined by *AP2* and *Crabp1* expression studies (54).

All considered, these data suggest that multiple signaling pathways interact to direct the r4 neural crest stream into the second arch, and that these cells are able to translocate *Hoxa2* expression to the first arch if misdirected. Furthermore, distal/lateral middle ear elements are less impacted by the mutations described above, suggesting that different mechanisms are involved, as well as local signals required in their patterning. How then are mesenchymal condensations patterned once neural crest cells have migrated to the arches?

6. MESENCHYMAL CONDENSATION

Epithelial-mesenchymal interactions precede the onset of condensation. Endochondral ossification requires the formation of a mesenchymal condensation, occurring

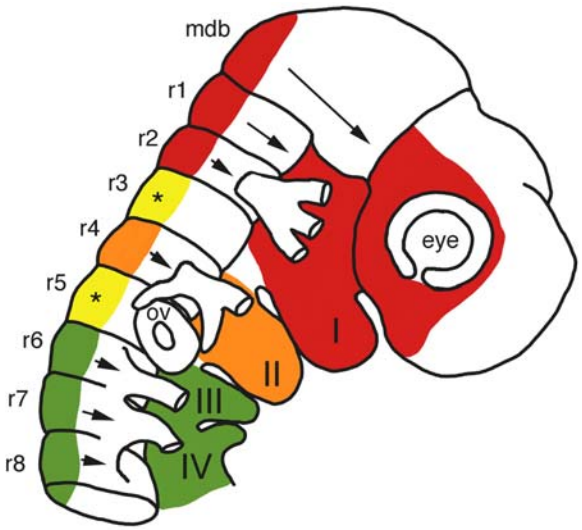

Figure 4. Origin of pharyngeal arch derived cranial neural crest. A line drawing with color-coded contributions from the rhombomere derived neural crest streams. Pharyngeal arches denoted by roman numerals. r3 and r5 crest (asterisks) provide a minimal contribution to the pharyngeal arches, mostly undergoing apoptosis. Middle ear skeletal elements arise from the proximal region of the pharyngeal arches in chick and mouse. Abbreviations: mdb, midbrain; ov, otic vesicle; r, rhombomere.

due to the aggregation of neural crest cells. The number of cells and duration of the condensation phase in part determine the size of the condensation, although the exact mechanism of condensation has not yet been elucidated (44, 55-57). Similarly, the shape and re-shaping of condensations to form individual skeletal elements requires further study, with examination of crucial extracellular matrix and cell surface interactions. Discussed below are some of the tissue and molecular interactions known to play a role in condensation.

In chick, endoderm transplanted homotopically from quail to the second arch induced the caudal basihyal and basibranchial of the tongue hyoid-derived elements. More lateral grafts induced ceratobranchial and epibranchial tongue cartilages. Ablation had the opposite effect, with loss of these cartilage elements (58). The effect of these grafts on the columella was not evaluated, but it seems likely that pharyngeal endoderm has an important role to play in its patterning. The columella forming mesenchyme is situated at the level of the proximal second arch, at the lip of the dorsal and ventral pharyngeal endoderm, with hyoid mesenchyme more distally within the arch (2). In chick, Fgf8 signaling patterns the Hox-negative first arch elements, but the signals required from the second arch endoderm are unknown (34). The identity of foregut endoderm is altered in VAD embryos. VAD embryos do not survive beyond day 4, and as a result, the effect on the middle ear cartilages that are identifiable by Alcian Blue staining at day 7, cannot be assessed.

Hoxa2 is a selector gene and is the most anteriorly expressed Hox gene (r1/2 boundary), with a critical role in the identity of r4-derived second arch mesenchyme. The temporal role of *Hoxa2* is complex. *Hoxa2* is downregulated in r1/2 crest during migration, and thus, second arch crest-derived mesenchyme, derived from r4, is the most anterior limit of Hox expression in the arches (59). Homeotic transformation and strongly hypoblastic skeletal elements form after aberrant rostral expression of *Hoxa2*, the only Hox gene able to do so in the arches, with the first arch taking on and partially duplicating second arch identity (60-62).

The knockout of *Hoxa2* in mouse has the reverse effect, with second arch elements transformed into first arch-like fates, with mirrored polarity. The stapes and other hyoid elements fail to form in these mutants (63, 64). The *Hoxa2*$^{flox/flox}$*;Wnt1-Cre* mutant phenocopies the conventional mutant, with malleus, incus, and tympanic ring duplication, and altered gonial and hyoid bones (65). Temporal analysis indicates a temporary inhibitory effect on dermal bone formation by *Hoxa2*, which also represses *Cbfa1/Runx2* osteogenic marker expression (66). This ability to induce homeotic transformations between first and second arches is suggestive of a shared ground pattern, in which *Hoxa2* represses a number of signals in the second arch cells, whereas in the first arch, the patterning mechanism is unrestricted (64, 67).

Particularly of note, *Hoxa2* normally inhibits several first arch genes that are activated in the second arch in mutants: *Ptx1* (52); *Six2*, a direct downstream target of *Hoxa2* (68); and, *Lhx6*, *Alx4* and *Bapx1*, with concomitant downregulation of second arch *Msx1* (65). *Six2* functional analysis showed that *Hoxa2* directly binds the *Six2* promoter, resulting in suppression of *Igfbp5* and increasing *Igf1* expression in the second arch. Moreover, mutation of Six2 results in a partial rescue of the Hoxa2 phenotype in compound mutants (69).

In the chick, overexpression by electroporation leads to Meckel's cartilage and the quadrate transforming to form duplicated tongue skeleton elements (70). This change in fate requires global *Hoxa2* overexpression in crest and surrounding tissue. First arch targeting alone is insufficient to induce second arch elements, supporting the hypothesis where neural crest is partially pre-patterned, requiring local cues for further patterning. Expression persists in the neural crest during arch patterning and later cartilage differentiation, but downregulates as ectomesenchyme condenses (66). Other Hox genes are no longer expressed at these stages. *Hoxa2*, therefore, has an effect during differentiation, not migration, and patterning of neural crest is determined *in situ*. Of note, Gsc is ectopically expressed and maintained, even though there is only partial overlap with Hoxa2, and in null mutants *Gsc* is unaffected.

What other signals are required for positional identity and patterning of mesenchymal condensations? Mutations affecting the size or shape of condensations have important implications for the derived skeletal elements. First arch ectomesenchyme gives rise to a single

condensation that will form both malleus and incus (44). So how is development regulated, giving rise to separate elements from a single condensation?

Six *Dlx* transcription factors regulate the proximo-distal identity of the first and second arches (71). *Dlx* expression is arranged in a nested pattern, with overlapping bi-gene pairs, proximal to distal, in the first and second arch ectomesenchyme. *Dlx1/2* are expressed proximally, with *Dlx5/6* expression detected more distally, and *Dlx3/4* expression is restricted to the distal-most portions of the arches. The stapes of *Dlx1*$^{-/-}$, *Dlx2*$^{-/-}$ and *Dlx1/2*$^{-/-}$ embryos is abnormally small, often lacking the stapedial foramina, and in *Dlx2*$^{-/-}$ and *Dlx1/2*$^{-/-}$ embryos, does not articulate with the incus. *Dlx2*$^{-/-}$ and *Dlx1/2*$^{-/-}$ embryos have an enlarged incus, fused to an abnormal basisphenoid (72). The gonial and anterior processes of the tympanic bones are slightly smaller and truncated, respectively (71). *Dlx5*$^{-/-}$ embryos show defects in the malleus, which is caudally extended and thickened at the manubrium. A smaller malleal head is observed, and the tympanic ring is smaller and thicker (71). *Dlx5/6*$^{-/-}$ double mutants have a transformed malleus resulting in a dysmorphic cartilage structure fused to a similarly affected incus. Interestingly, genes participating in maxillary arch development were maintained (*Dlx1/2, Msx1/2* and *Prx1*), whereas mandibular development genes (*Alx4, dHAND, Dlx3, Dlx5/6, Bmp5* and *Pitx1*) were all absent. Depew and coworkers have extensively reviewed *Dlx* and *Dlx* compound mutations (71).

The Prx1 homeobox gene is expressed in mesenchyme surrounding prechondrogenic condensations, and may be active in establishing chondrogenic fate. Two isoforms, Prx1a and Prx1b have been identified, but individual expression pattern data for pharyngeal arches has not been analyzed. In limbs, Prx1a is expressed in early development, with Prx1b expression subsequent to condensation. Prx1 modulates cell proliferation where Prx1a stimulates proliferation, in contrast to Prx1b promoting increased apoptosis (73).

Prx1 inactivation in mice affects all three ossicles. A hypomorphic malleal manubrium and processus brevis are observed, with the latter displaced and the incus fused to the quadrate. It is conceivable that the lack of dermal bone formation in these embryos leads to increased cell numbers that are converted into the extra cartilage, fusing to the incus. The gonial and tympanic bones are missing. In the second arch there is no stapedial foramen, and it remains fused to Reichert's cartilage (74).

As expected, BMPs are required for chondrogenesis, with gain-of-function mutants increasing proliferation, leading to fused skeletal elements, whereas loss of BMP receptor function leads to severe loss of cartilage. Dynamic Bmp2, Bmp4 and Bmp7 expression in the mesenchyme surround each condensation, with *Gdf5* expressed in the joint regions and *Nog* expression in the chondrocytes (75). In *Nog* mutant embryos the ossicles were overgrown and fused with no joints, making identification of individual elements difficult. In heterozygote *Nog* mice, the stapes had an ectopic bone bridge fused to the styloid process, potentially due to failure of these two elements to separate correctly. A balance between BMP and NOG levels is required, with NOG normally binding and blocking *Bmp4* and *Gdf5* expression (75). *Bmp5* mutants also have a malformed stapes with fused crura, and a short ear phenotype due to smaller than normal pinna (76). BMP signaling is thus not all or nothing and the role of ligands, receptors and inhibitors still requires clarification.

The terms condensation and chondrogenesis are often used interchangeably, but the latter is a separate process dependent on the former. Once condensation has occurred, the inhibition on chondrogenesis is released and cartilage begins to form, as is next discussed.

7. CHONDROGENESIS AND OSTEOGENESIS

Chondrogenic cells initially form immature, proliferating chondrocytes, maturing to form prehypertrophic and then enlarged terminal hypertrophic chondrocytes. Finally, osteoblasts replace the cartilage matrix to form ossified tissue. In chick, chondrogenesis occurs at day 7 (2, 26). Pre-chondrogenic cells express *Sox9,* and with the onset of chondrogenesis *Col2* is expressed. BMP signaling first promotes proliferation and then chondrocyte differentiation, with roles in *Sox9* maintenance and induction of *Coll2* and *Col10*-dependent matrix production. The downregulation of cell adhesion molecules appears to be a necessary prerequisite to condensation (44). Loss of the functionally redundant *Smad1* and *Smad5* genes blocks the BMP signal cascade and thus endochondral ossification. FGF and BMP signaling act antagonistically with FGF downregulating *Bmp4* and *Ihh*, expressed in prehypertrophic cells. The *Ihh*/*Pthrp* feedback loop also regulates chondrocyte proliferation and differentiation (77).

Bapx1/Nkx3.2 is expressed in immature, proliferating chondrocytes and is downregulated, then finally extinguished during maturation from prehypertrophic to hypertrophic chondrocytes. Overexpression results in delayed maturation; and, in loss-of-function mutants, premature maturation. This effect is due in part to reciprocal expression patterns, where *Bapx1* represses *Runx2*, the expression of which leads to hypertrophy and ossification. In chick limb experiments, rescue of chondrocyte maturation occurred when RCAS-Runx2 was introduced in tissues infected with RCAS-Bapx1. The extent to which this mechanism is involved in the middle ear is unknown, however, *Runx2*/*Cbfa1* expression is adjacent to *Bapx1* in the tympanic and gonial condensations, suggesting a role (78). Moreover, absence of *Bapx1* leads to loss of the gonium, anterior tympanic ring and an abnormal malleus, all regions that normally express Bapx1. Bapx1 does not affect incudo-malleal joint, although expression is detected in at the periphery, where articulation occurs (29).

In the next section, genes involved in formation of individual skeletal elements are discussed.

8. SKELETAL ELEMENTS: OSSICLES

8.1. Columella

The columella is a single ossicle consisting of a proximal ossified columella shaft and footplate inserted into the oval window. Distally, toward the tympanum, the persistent cartilaginous extracolumella has three processes: the supracolumella; extracolumella; and, infracolumella. There is a paucity of gene expression data available with specific reference to the columella in amphibians and reptiles, with the most data available in chick.

In the direct developing anuran, *Eleutherodactylus coqui*, the expression of *bmp4*, *col2a1*, *runx2* and *sox9* have been described for cranial skeletal elements. In *Xenopus laevis*, runx2 and col2a1 expression data have similarly been described (79-81). However, the columella has not been examined in detail.

Peanut agglutinin lectin (PNA), a marker of prechondrogenic cells, reveals the position of the proximal second arch mesenchyme at stage 18 (day 3). At this stage, the mesenchyme is in direct contact with the pharyngeal endoderm of the first pouch. As early as stage 24 (day 4), the tissue is beginning to separate from the more distal hyoid mesenchyme and condense. By stage 30 (day 6.5), the columella is a separate, condensed structure, with chondrogenesis imminent (2). Changes in Bmp2, Bmp4, Col1, Col2, Ihh and Sox9 marker gene expression reveal the timing of the successive processes in endochondral ossification (2). Sox9 is expressed in prechondrogenic cells and downregulated at day 9. Bmp2 and Col2 are expressed at day 7, with downregulation of Bmp4 as chondrogenesis occurs. Ihh is detected in the columella only at day 10, and Col1 in the forming perichondrium of the shaft and footplate from day 13 (2).

Morphologically, first three and then five chondrogenic centers are initiated within the columella condensation, which already has a distinctive shape by day 7.5. A double-layered (inner chondrogenic and outer fibrous) perichondrium surrounds the columella at day 10. Col1 expression at day 13 indicates the onset of ossification within the bony collar/periosteum of the columella shaft. Alizarin Red S staining of the bony collar is detectable at day 13.5. A separate region of calcification develops on the medial surface of the footplate at day 14, before the two regions merge by day 15. ColX is not detected in the ossicle at day 12, but upregulates when cavitation begins on day 16 in the shaft tissue during chondrocyte maturation once hypertrophic chondrocytes have formed (82). Ossification continues distally in the shaft, with blood vessels and marrow sinuses visible by day 18. The columella hollows out through resorption of bone, with an endosteum as the single cell layer lining the internal surface of the bone and cavity. The annular ligament is situated in the oval window, and to accommodate growth during fetal and post-hatching development, the footplate retains an area of proliferating and hypertrophic cells. The joint region between bone and cartilage forms an asynchondrotic joint at 42 days post-hatching (2, 25, 26, 82). The ratio of tympanic membrane to footplate doubles post-hatching from 11:1, with a 4-5 times lever ratio to amplify and transmit sound (2, 27).

8.2. Mouse ossicles - malleus

The malleus has several components. The main two constituents are the malleal head, which articulates with the incus, and the manubrium, which inserts into the tympanic membrane, separated by the neck, with anterior and lateral (brevis) processes. The inferior end of the manubrium inserts into the tympanic membrane at the umbo. The manubrium develops between E12.25 - E13.25, with full development of the cartilage by E14.5. Extracting the manubrium for culture arrests its development at the stage it was dissected, suggesting that the mesenchyme itself does not contain all the signals required to develop, and furthermore, that sequential signaling occurs, where the later the stage the manubrium was dissected, the more developed it appeared (28). Development of the manubrium and tympanic ring requires a functioning external auditory meatus, with development of the manubrium, tympanic ring, and membrane temporally coordinated.

Dlx5-Msx5 double knockout mice display compound effects with a deformed malleus and strut. *Dlx5* is normally expressed in the malleus and incus, with the single knockout retaining the strut. The *Msx1* homeobox gene is expressed in both first and second arches, with lower levels in the posterior of the first arch, opposing higher levels in the anterior of the second arch. In the *Msx1* single knockout mutants, the second arch expression level is reduced to a first arch level, and is extinguished in the first arch, resulting in a deformed malleus with absence of the process brevis, but other structures are unaffected (65, 83, 84).

8.3. Incus

A body, short, and long processes comprise the three main elements of the incus. The head articulates with the malleal head forming the incudo-malleal joint. The lenticular process, at the bottom of the long process, articulates with the stapes. The short process attaches to the middle ear cavity wall via the posterior incudal ligament. Reichert's cartilage gives rise to both the stapes and the lesser horn of the hyoid bone.

Endothelin1 is the ligand for the endothelin-A receptor, with mutation disrupting middle ear formation (85, 86). A requirement for endothelin signaling is demonstrated in *Ednra*$^{-/-}$ embryos, where the malleus and incus are missing, and small, undefined cartilage nodules are present (87). A recent study details a conditional knockout strategy targeting the *Ednra* allele (*Ednra*^{fl}), crossed with a *Wnt1-Cre* transgenic strain, *Ednra*$^{fl/fl}$*;Wnt1-Cre* (86). Skeletal analysis reveals that 25% of embryos lack the tympanic and gonial bones and show severe reduction or occasional absence of the malleus and incus. The stapes, otherwise apparently normal, is occasionally attached to the greater horns of the hyoid, with the hyoid bones fuse with the basisphenoid. The remainder exhibited an enlarged malleus, and a malformed incus articulating via an ectopic bone with the pterygoid. Temporal application of an endothelin antagonist reveals progressively more

severe dysmorphogenesis of first arch derivatives when applied between E8.0 and E9.5 (86).

8.4. Stapes

The stirrup-shaped bone in mammals consists of four components: the head; anterior and posterior crura; and, the footplate, with a central foramen for the stapedial artery.

Brn4/Pou3f4 mutants exhibit a flattened stapedial footplate with a disturbed oval, being more pointed at one end, possessing a thinner crus in affected embryos (88). The result of this defect is compromised middle ear conduction (89). Inappropriate bone remodeling results in fixation of the stapes within the oval window, and is a key feature of otosclerosis.

8.5. Tympanic ring and gonial bone

The tympanic ring surrounds, supports, and anchors the tympanic membrane and is attached by the gonial bone to the skull. Both bones are first arch neural crest-derived and undergo dermal/membranous ossification. *Tbx1* (53) and *Dlx5* (90) mutants only mildly affect the tympanic membrane, with a shorter, thicker tympanic ring. In *Hoxa1* null mutants, the tympanic ring is displaced rostrally (91), whereas *Hoxa2* mutant mice have a duplication, featuring an altered gonial bone (65). *Bapx1* and *Gsc* have partially overlapping expression, but function independently in tympanic ring specification. *Gsc* is required for tympanic ring development. *Bapx1* functions in patterning the gonium and anterior ring, with mutants losing both tympanic ring and gonium. Formation of the manubrium, tympanic ring, and membrane are temporally coordinated for proper development. Importantly, a functional external auditory meatus is essential to development of the tympanic ring (29).

8.6. Otic capsule

The otic capsule surrounds and protects the inner ear. The footplate of the columella or stapes is inserted into the oval window. The annular ligament holds the footplate against the oval window, with movement of the footplate stimulating endolymph and thus the basilar membrane of the cochlear duct. Signaling from the otic epithelium to the adjacent periotic mesenchyme leads to condensation of the capsule.

Chick otic capsule has a composite origin. The paraxial first somite mesoderm forms the majority of the pars canalicularis, a contribution from head mesoderm to the pars cochlearis, with the remainder of the walls of the dorsal (pro-otic) pars cochlearis derived from the neural crest (3, 45, 92).

Wnt pathway signaling plays a key role in otic capsule morphogenesis. Wnt gene expression studies detected the Wnt Frizzled receptor, Fzd8, ventral to the otocyst at stage 22, with Fzd1, Fzd7, Fzd9, Fzd10 expression at stage 24-26. Wnt ligands appear to be restricted to the otocyst, with antagonists Frzb and sFRP1 also detected at these stages. By 8-10 days, Fzd9 is excluded from the otic capsule and is only detected in otic epithelium and surrounding mesenchyme. Frzb, Wnt9a and Fzd7 are all expressed in the otic capsule (93). Unsurprisingly, Sox9 is expressed in the otic capsule, being detected from day 5. Col2 is upregulated at day 7, at the onset of chondrogenesis (2).

Regarding the mouse otic capsule, exogenous *Wnt5a* stimulates otic capsule chondrogenesis. *sfrp3* in the inner ear is able to interfere with Wnt5a signaling, resulting in suppression of chondrogenesis, similar to *Wnt5a* null embryos. Thus, it appears that *sfrp3* and *Wnt5a* act antagonistically in controlling chondrogenesis within the otic capsule (94).

Otic capsule remodeling is important for formation of the oval and round windows. High levels of osteoprotegrin when secreted into the cochlear perilymph can diffuse into the otic capsule and inhibit remodeling of the otic capsule. Mechanistic studies on insertion of the footplate into the oval window are lacking, although changes in the oval window have been noted in *Prx1*, *Prx2* and *Hoxa2* mutants. The columella/stapes condensation is thought to induce formation of the oval window. In Hoxa2 mutants both the oval window and stapes fail to develop. However, in some RA mutants, even in the absence of a recognizable stapes, the otic capsule has a structure resembling the oval window (61, 63, 64, 95, 96). Thus, interaction between the footplate and otic capsule mesenchyme is an area for future study. Given the clinical importance of otosclerosis, in which the stapes becomes fixated in the oval window, knowledge of signaling and tissue morphogenesis of the otic capsule/oval window, and stapes interactions are vital to improved therapeutic outcomes.

9. OTHER COMPONENTS OF THE MIDDLE EAR

9.1. Tympanic membrane and external auditory meatus

The trilaminar tympanic membrane consists of three layers: an outer, first cleft derived ectodermal layer; an inner first pouch pharyngeal endoderm epithelium; and, a fibrous (collagen) mesenchymal middle layer (lamina propria) sandwiched in between (97). The tympanic membrane acts like the skin on a drum, vibrating when stimulated by sound, and transmitting these airborne vibrations via the mechanical chain ossicles to the inner ear (28). Unlike mammals, which have two components to the membrane, the pars tensa and pars flaccida, the chicken has only the pars tensa. The pars flaccida consists of elastic collagen fibers, whereas the pars tensa in both mammals and avians contains radial and circular inelastic collagen fibers, detectable by day 12 in chick embryos (2, 26). The auriculotemporal nerve innervates the external tympanic surface.

Maturation of the tympanum occurs beyond the fetal stage with expansion of the surface area from over 100% to nearly 300% in increasing increments in chick, mouse, gerbil and rat (97). From hatching to P74, functioning of the tympanic membrane improves, with collagen fiber density and thickness of the central lamina propria region increasing. When chickens are tested at one

year, only slight improvements in function are detected. The thickness of the ectoderm and endoderm layers is essentially unchanged after day 11, indicating their maturation occurred during embryogenesis.

Invagination of the external auditory meatus is controlled by the dermal-derived tympanic ring, which in turn anchors and stabilizes the tympanic membrane. Epithelial signals (*Fgf8*, *Endothelin1*) induce underlying mesenchymal cells to express *Gsc* and *Prx1*, which form the tympanic ring. *Gsc* and *Prx1* mutants have aplasia of the external auditory meatus, tympanic membrane, and acoustic cavity, and the malleal manubrium is hypomorphic. Early *Gsc* expression in the external auditory meatus is required for induction of condensation, whereas later condensation is cell autonomous. (74, 98-101).

Recombination of the external auditory meatus and first arch mesenchyme in culture results in induction of *Sox9* and *Prx1* expression. *Sox9* has expression in the malleal manubrium condensation and is complimentary to *Prx1*, which is also in the mesenchyme, but excluded from the *Sox9* positive manubrium condensation. Both are required for the development of the manubrium and tympanic ring (28). Notably, these condensations always form at a distance from, and never in direct contact with, the ectodermal epithelium (102).

A mirror image duplicated of the ring occurs in *Hoxa2* mutant embryos (63, 64) and the ring is lost in embryos treated with RA, which also fail to form an external auditory meatus (103). Loss of endothelin-1 or its receptor leads to loss of first arch structures, including the malleus, incus, and tympanic ring (85, 87). *Bmp4*, *Fgf4* and *Fgf9* are expressed in the meatus, although a significance role for these signaling molecules remains to be established (28).

9.2. Middle ear cavity and Eustachian tube

The middle ear cavity is located in the petrous portion of the temporal bone. Pharyngeal endoderm outpouching from the pharynx forms the tubotympanic sulcus (later called the Eustachian tube). The endodermal epithelium forms a continuous sheet from the pharynx, through the Eustachian tube, and finally the lining of the middle ear cavity. Notably, the lumen of the Eustachian tube in chick is lined with ciliated pseudostratified columnar epithelium and secretory cells by day 16/17, consistent with the characteristics of respiratory epithelium. The epithelium of the middle ear cavity itself consists of poorly differentiated endoderm (nonciliated cuboidal cells), which does not mature until after hatching.

The mesenchyme that filled the middle ear cavity and originally surrounded the early skeletal elements gradually disappears through a process of cavitation. How this clearing occurs is not understood. The expanding epithelial lining is incapable of pushing the mesenchyme aside. Apoptosis does not appear to play a large role in mouse, and appears to be absent in chick middle ear (26).

The lining of the middle ear and mastoid cavities is particularly susceptible to inflammation. Otitis media has been extensively reviewed elsewhere, and will not be covered here, but interestingly the cells change shape and in some cases are transformed into a mucus-producing population. Thus, it is possible that otitis media and failure of cavitation are related. Several syndromes where there is poor clearance of cells in the middle ear cavity demonstrate this possibility, Down's Syndrome being a well-known example (104).

9.3. Muscles and nerves

Two muscle tendons, the tensor tympani and stapedius muscle are correctly located by neural cells in the insertion site (35), even though they are derived from cranial paraxial mesoderm (37). The tensor tympani muscle body is enclosed in its own temporal bone cavity in an anterior-to-posterior plane. The tensor tympani tendon lies perpendicular to the muscle body, and attaches to the malleal manubrium. Contraction displaces the malleus and tympanic membrane medially, increasing the tension on the tympanic membrane. Similarly, the stapedial tendon, which attaches to the head of the stapes with a perpendicular muscle body (superior-inferior plane), inhabits its own temporal bone cavity. The two nerves that pass through the middle ear cavity do so without innervating any part of the middle ear. They are the horizontal portion of the facial nerve (CNVII), which passes superior to the stapes, and the chorda tympani, a recurrent branch of the facial nerve, which passes medially to the malleus neck (37).

9.4. Joint formation

Treacher Collins and Branchio-Oto-Renal (BOR) mutants display a fused malleus and incus leading to conductive hearing loss (30, 31). BOR syndrome results from *Eya1* and *Six1* mutations, with malleal-incudo fusion. *Gdf5* and *Bapx1* upregulate before any overt sign of joint formation, and during joint formation at day 14 *Sox9* and *Col2* are downregulated in the interzone. *Gdf6* expression is only detected following joint formation.

Emx2 is expressed in the skeletogenic neural crest and is required for formation and the relative position of the skeletal elements, including the incus and articulating surface of the malleus, which is lost in mutants (105). Heterozygous mutant mice display defects in the incudo-malleal joint due to altered articulatory surfaces. Instead, the stapes and incus are separated and homozygote mutants display a more severe phenotype with loss of the incus, and complete loss of the articular surface of the malleus (105).

Interestingly, Guinea pig and Chinchilla have a single malleal-incudo complex. In the guinea pig, a joint begins to form to separate the two elements, but is arrested before cavitation, resulting in a single complex. *Eya1* and *Six1* loss in the mouse leads to a similar fusion, but the guinea pig has normal levels of *Eya1* expression, indicating that the signaling mechanism is more complex than simple loss of expression of *Eya1* and *Six1* (31).

10. CONCLUSIONS

Numerous studies of mouse mutants describe middle ear defects, without the middle ear necessarily

being the main focus of the study. There is a growing body of evidence describing the morphological development, induction and patterning mechanisms of the external and middle ear hearing apparatus, providing a unique organ system for study. The middle ear as an organ system will aid our understanding of molecular mechanisms and tissue interactions during development. Although non-mammalian model systems have a single stapes/columella bone, which is the developmental equivalent of the mammalian stapes, elucidating the patterning of the apparatus as a whole will benefit from their study. Moreover, translation of basic knowledge to the clinical setting will have therapeutic benefits to those with conductive hearing loss and thus to society, beyond just understanding normal and abnormal development.

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