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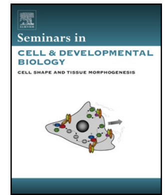

Sculpting the labyrinth: Morphogenesis of the developing inner ear

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ABSTRACT

The vertebrate inner ear is a precision sensory organ, acting as both a microphone to receive sound and an accelerometer to detect gravity and motion. It consists of a series of interlinked, fluid-filled chambers containing patches of sensory epithelia, each with a specialised function. The ear contains many different differentiated cell types with distinct morphologies, from the flask-shaped hair cells found in thickened sensory epithelium, to the thin squamous cells that contribute to non-sensory structures, such as the semicircular canal ducts. Nearly all cell types of the inner ear, including the afferent neurons that innervate it, are derived from the otic placode, a region of cranial ectoderm that develops adjacent to the embryonic hindbrain. As the ear develops, the otic epithelia grow, fold, fuse and rearrange to form the complex three-dimensional shape of the membranous labyrinth. Much of our current understanding of the processes of inner ear morphogenesis comes from genetic and pharmacological manipulations of the developing ear in mouse, chicken and zebrafish embryos. These traditional approaches are now being supplemented with exciting new techniques—including force measurements and light-sheet microscopy—that are helping to elucidate the mechanisms that generate this intricate organ system.

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1. Introduction

In any study of organogenesis, it is important to gain an appreciation not only of the genetic control of patterning but also of the morphogenetic events that give rise to the three-dimensional form of the mature organ system. Understanding the coupling of signalling pathways and transcription factor network activity to the cell behaviours and physical forces that effect these morphogenetic events is thus one of the major challenges in the field. The development of new technologies, particularly in live imaging, is now opening up new possibilities for tackling these challenges. For the inner ear, such studies have clinical relevance: congenital hearing loss can also be accompanied by some form of morphological

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anomaly, such as Mondini dysplasia (incomplete partition or coiling of the cochlea). Aplasia of the semicircular canals or whole labyrinth can also cause severe disruption of vestibular function.

In this review, we consider a selection of the morphological rearrangements that take place as the inner ear develops. We focus on four topics: formation of the otic placode and vesicle; neurogenesis and generation of the VIIIth ganglion; segregation of sensory epithelia; and formation of the semicircular canal ducts. We have omitted discussion of a number of other important processes, including formation of the endolymphatic duct and sac, establishment of the precise cytoarchitecture of the mammalian organ of Corti, the role of surrounding tissues (including hindbrain and periotic mesenchyme), and the morphogenesis of ancillary structures, each of which would justify a separate review in its own right. We end with a perspective on the new methodologies that are pushing the boundaries of our understanding of how patterning is coupled to morphogenesis in the developing inner ear.

2. Segregation of the otic placode from the pre-placodal region (PPR)

2.1. Cell movements

The otic placode together with other cranial placodes (adenohypophyseal, olfactory, lens, trigeminal, epibranchial, otic and lateral line) and the neural crest give rise to the elements of the cranial peripheral nervous system. The cranial placodes do not develop directly as individual entities from the ectoderm but emerge from the common pre-placodal region (PPR), a horseshoe-shaped subdomain of the ectoderm adjacent and lateral to the neural plate and neural crest [1–5]. The PPR expresses a combination of transcription factors of the Six1/2, Six4/5, Dach, Eya, Dlx, Gata, and Foxi families that confer its identity and competence for specific placode-inducing signals [6–14]. The latter, by acting upon the PPR precursors, drive the splitting of the PPR and emergence of individual placodal fates [15–17]. The segregation of the PPR into placodes is progressive. At the level of the hindbrain for example, prior to the appearance of the otic placode, a large *Pax2/8*-expressing domain encompasses the precursors of future epibranchial and otic placodes (also lateral line precursors in anamniotes). This domain has been coined the otic-epibranchial precursor domain (OEPD) to highlight the close developmental relationship between these placodes [9,10,18–20]. The inductive events involved in the development of the OEPD and otic placode are reviewed elsewhere in this issue [21]; we focus here on the morphogenetic movements leading to the segregation of the large PPR into discrete placodes within the cranial ectoderm.

Fate mapping of pre-placodal precursors in chick indicates that otic precursors are interspersed with future neural tissue, neural crest and other placodal cells until the four-somite stage; extensive cell movements have been observed to accompany placode development, enabling the segregation of the different cell types [22]. At early stages (stage 5–6 in chick), otic precursors were found over a large territory of the PPR, at the level of rhombomeres 2–7 of the hindbrain, but were then progressively restricted to form the otic placode at the level of rhombomeres 5–6. Convergence of lateral cells to medial positions was the most dramatic cell movement, accompanied by splitting and cell mixing between groups of cells. In zebrafish, live imaging of cells expressing GFP driven by the *pax2a* promoter within the OEPD showed that most GFP-positive cells converge from anterior, posterior and lateral positions to form the otic placode, but more anterior and posterior GFP-positive cells also contribute to epibranchial ganglia [23]. Analysis of Pax2a protein expression, together with heat-shock-induced mis-expression and morpholino-based gene knockdown, demonstrated that cells with high levels of Pax2a protein have a tendency to contribute to the otic placode, while lower levels of Pax2a bias precursors to the epibranchial placodes [20]. Exactly how the levels of Pax2a can influence the sorting and/or convergence of pre-placodal precursors needs to be investigated further, but probably involves changes in cell adhesivity. Interestingly, a morpholino-based study suggests that directed cell movements and convergence of pre-otic precursors to form the zebrafish otic placode relies partly on the function of the extracellular matrix receptor Integrin-$\alpha 5$ [23].

Similar cellular movements leading to the segregation of intermingled anterior PPR precursors into the anterior cranial placodes (olfactory, lens, adenohypophyseal, trigeminal) have also been described [3,24–26]. The extent of the directional cell migration differs between species; in *Xenopus* and zebrafish, cell movements are restricted to small areas and no large-scale cell sorting is detected [17,20]. Differences could be related to the species or to the periods in which movements have been analysed, which are limited prior to placode coalescence. When cells of the OEPD display small-scale movements, they contribute to distinct otic regions depending on their initial anteroposterior location in the OEPD, the most anterior cells being preferentially allocated to the anterior neurogenic domain and statoacoustic ganglion (SAG) [20] and not to the posterior domain of the inner ear. In conclusion, while migratory movements have recently been followed in real time, the underlying molecules involved in the chemotaxis, sorting and collective movements are little known. Moreover, it is still debated whether PPR cells are already lineage-restricted before their sorting out or whether random movements favour their position along the anteroposterior axis, followed by the reception of distinct signals that direct cells to specific placodal fates (see also [27]).

2.2. Placode formation

2.2.1. Coalescence into a placode

How do placodes appear as a cluster of cells after the segregation of PPR cells? Compared with other placodes, morphogenetic events of otic placode formation have received scant attention, but studies on other placodes hint at generic mechanisms. Whitlock and Westerfield have shown that before the final appearance of the olfactory placode, olfactory precursors extend over a long and thin territory that progressively converges to a shorter and wider domain along the anteroposterior axis and mediolateral axis respectively [24]. A similar event takes place during the coalescence or convergence of the otic placode. Alvarez and Navascués found long cytokinetic bridges during this process, and have proposed a link between cellular displacements after mitosis and placode formation [28]. In line with this, recent imaging of convergence movements during chick gastrulation identified mitosis as a driver for epithelial rearrangements [29]. This highlights the need to re-evaluate, with modern imaging techniques, the contribution of cell division orientation and shapes to otic placodal morphogenesis.

Other tissues also impact on placodal development. In particular, coalescence and positioning of the zebrafish olfactory and epibranchial placodes are linked to migration of the adjacent neural crest [30,31]. The latter influences the timing of establishment of a basal lamina surrounding the olfactory placode and the neural tube that segregates both populations and favours condensation of the olfactory placode. During formation of the epibranchial placodes in chick, coordinated cell migration between placodal and neural crest cells results from a "chase and run" cellular behaviour, in which neural crest cells chase placodal cells and then placode cells run away as they are contacted [32]. The classical chemokine system Sdf1-Cxcr4, known to underlie various migratory events (for example, migration of germ cells and lateral line primordia) is involved in the coordinated placode-neural crest migratory behaviour [32]. Epibranchial placodal cells express the ligand *Sdf1* and remain in

close association with each other through expression of high levels of N-cadherin, while the neural crest cells express the chemokine receptor *Cxcr4* and establish transient cell–cell contacts with the placodal cells. This "chase and run" mechanism is thought to contribute to the final position of the placode and the coalescence of epibranchial precursors into a defined placode. It remains to be investigated whether a similar interaction occurs between the otic placodal precursors and the surrounding neural crest, but the lack of expression of *Sdf1* in the zebrafish and chick otic placode suggests that other mechanisms might be involved in coalescence of the otic placode [33,34]. Eph/ephrin signalling, while not tested, is a good candidate. Several members of this signalling pathway are expressed at otic placode and vesicle stages, as well as in surrounding tissues [35]. It remains, thus, to be characterised whether Eph/ephrins mediate sorting of otic placodal cells and/or segregation from the surrounding OEPD cells, as happens during hindbrain development [36].

2.2.2. Placodal thickening and interkinetic nuclear migration

After the coalescence of otic precursors, the chick and mouse otic placode becomes visible and distinct from the surrounding non-placodal ectoderm as a thickened region [37]. While a 'thickening' describes cranial placodes of amniotes (chick and mouse) well, where presumptive otic cells transit from a squamous-cuboidal to a columnar shape, in zebrafish, the otic placode appears to emerge from the unorganised ectodermal cells beneath the enveloping layer (EVL) as a compacted mass of cells, and the thickening is less obvious (Fig. 1). In both cases, the emergence of the morphologically visible otic placode is concurrent with the elongation of cells and the acquisition of a prominent apicobasal polarity. In amniotes this takes place in a 2D sheet of cells, while in zebrafish, the medial otic cells in close contact with the hindbrain epithelialise before the lateral cells [38,39]. Whether extrinsic signals emanating from the surrounding tissues direct the epithelialisation is unknown. Cellular elongation in the otic placode is also concomitant with the initiation of interkinetic nuclear migration (IKM), a process typical of neurogenic epithelia that describes the dynamic oscillatory movement of nuclei within the elongated cells. In epithelia undergoing IKM, nuclei are observed at different apicobasal positions dependent on the phase of the cell cycle, giving the tissue a pseudostratified appearance [40]. Prior to cell division, nuclei move towards the apical side; the cells round up at the apical surface of the epithelium, enter mitosis and divide. Pseudostratification allows greater cell density of the epithelium [41], which has been suggested to promote rapid tissue expansion [42]. This is an interesting point, since the otic placode has a high mitotic index and its epithelial organisation would favour a rapid expansion of the organ. In foetal intestinal epithelium, cell elongation and acquisition of a pseudostratified epithelium depend on actomyosin and the actin-binding protein Shroom3 [43,44], which is known to be expressed in the *Xenopus* otic placode [45].

The signals that trigger cells to adopt a pseudostratified arrangement are unknown, but most probably are factors downstream of the otic placode inducing signals. A good candidate is the transcription factor *Pax2*, since it is expressed in all placodes, and its blockade by morpholinos in chick leads to the absence of N-cadherin and N-CAM, adhesion molecules that are necessary for the acquisition of columnar cell shape [46]. However, other transcription factors are

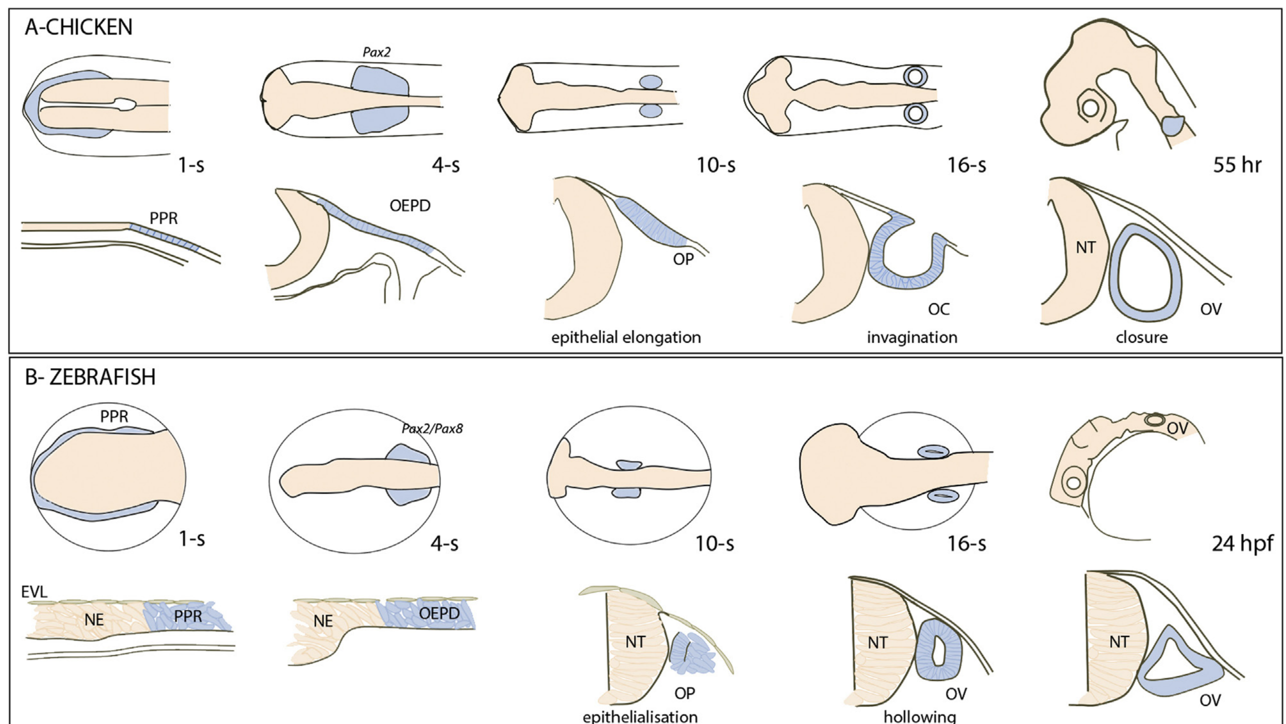

Fig. 1. Early development of the inner ear in chick and zebrafish A- In chick, the preplacodal region (PPR, blue) emerges as a horseshoe-shaped band adjacent to the neural plate at the 1 somite stage. By 4 somites, the PPR has split into larger preplacodal domains, including the otic-epibranchial domain (OEPD, blue) at the level of the hindbrain, which expresses *Pax2* and contains the precursors of the future epibranchial and otic placodes. The otic placode (blue) is morphologically visible as a thickening of the epithelium by 10 somites. At this stage, the epibranchial cells are segregated from the otic precursors. The otic placode starts to invaginate around 16 somites by a mechanism of apical constriction. Finally, the otic cup pinches off from the adjacent ectoderm to generate the otic vesicle underneath the ectoderm. B- In zebrafish, the PPR (blue) is also apparent by the 1 somite stage underneath the large cells of the enveloping layer (EVL). The OEPD expresses *Pax2* and *Pax8* and contains precursors for the anterior and posterior lateral line placodes, in addition to the epibranchial and otic precursors. Cells of the OEPD do not display a clear epithelial organisation yet. By 10 somites, placodal precursors coalesce into an unorganised mass of cells next to the posterior hindbrain. The otic placode appears by progressive epithelialisation from medial to lateral positions. Subsequently, the otic placode undergoes a process of hollowing, in which establishment of apicobasal polarity leads to the separation of the apical membranes and emergence of intercellular spaces that will be fluid-filled and expanded to generate the lumen. The otic vesicle with its central lumen is apparent by 18 hpf.

likely to be required for an otic phenotype, since ectopic expression of *Pax2* is not sufficient for the generation of ectopic placodes [46]. Spalt and/or Sox proteins could be those co-operative factors. Overexpression of *Spalt4* and *Sox3* in chick non-placodal ectoderm by electroporation is capable of generating ectopic placodal tissue [47–49].

Together with the acquisition of characteristic epithelial adhesion properties such as adherens and tight junctions [46,50], otic placodal cells also acquire an apicobasal polarity. By the otic placode stage, a distinct basal lamina composed of laminin, fibronectin and type IV collagen is already deposited at the basal side that separates the otic primordium from the adjacent neural tube in the chick [51]. Pard3, a member of the Par complex involved in establishing apicobasal polarity, becomes apically localised at the otic placode stage in zebrafish, together with the tight junction protein ZO-1 [38].

2.2.3. Invagination and hollowing

Most organs contain a cavity, at least during the initial phases of development, and the inner ear is no exception. After its formation, the otic placode undergoes a series of morphogenetic events that transforms the primordium into a 3D hollowed vesicle. In amniotes, the otic placode transits into the otic vesicle through an invagination event (formation of the otic cup), while in anamniotes, the spherical mass of placodal cells generates the otic vesicle by cavitation/hollowing (see below). Most probably there is a tight connection between the establishment of apicobasal polarity and otic vesicle formation, either by invagination or other mechanisms, since one of the drivers of chick otic invagination is F-actin, expression of which becomes enhanced at the apical domain of cells just at the invagination stage (13 somite-stage) [52]. A concentration of apical actin is also seen just before otic placode hollowing in the zebrafish [53]. It has been proposed that otic invagination in the chick embryo is biphasic. In the first phase, mesodermal action of FGF signalling at the basal side of otic placodal cells induces the phosphorylation of PLC-γ, which in turn leads to the activation of Myosin II. Myosin II activity then causes the depolymerisation of basal actin filaments and apical enrichment of these fibres [52]. This molecular cascade of events results in the basal expansion of the otic placode. The second phase involves apical constriction through the contraction of F-actin fibres, which causes cells to adopt a wedge-like shape, triggering tissue invagination as observed in many other organs [54,55]. It is plausible that Shroom3, which activates myosin II via Rock1 and Rock2 and promotes apical constriction during lens invagination [56], plays a similar role during inner ear invagination. During the phase of apical constriction, Myosin II, instead of promoting basal depolymerisation of actin fibres, drives the contraction of F-actin fibres at the apical side [57]. Changes in composition of the basal lamina have also been proposed to be involved in otic invagination by modulating the attachment of the otic placode with the underlying tissue [58]. It would be interesting to explore how the mechanical forces of adjacent tissues impinge on otic invagination. In mice, *Sox9* has been implicated in otic invagination by regulating adhesivity between cells downstream of *EphA4*, but whether the lack of invagination in *Sox9* mutants was a secondary effect of loss of epithelial integrity and mechanical disruption or reflected a direct role of *EphA4* in invagination was not tested [59].

In zebrafish, dynamical imaging of the events leading to otic vesicle formation shows that the process is similar to the previously described mechanisms of cord hollowing in zebrafish gut and secondary neurulation of the chick and mouse neural tube [60–62], in which intercellular spaces are generated at the apical side of cells; the cavity is not created by cell apoptosis. In the otic placode, two small cavities appear at the anterior and posterior poles, and subsequently extend in an unzipping mechanism to generate an elongated central lumen [38]. Two poles still form when anteroposterior signals are disrupted, as evidenced by the appearance of a lumen and positioning of hair cells in embryos lacking Fgf and Hh signalling [63], but it remains to be evaluated whether the identity or position of the two small initiating lumens is affected after inhibition of patterning cues. In chick, FGF signalling regulates the constriction necessary for otic invagination [52], but the role of Fgf signalling in otic hollowing in the zebrafish is not clear. In *fgf8*$^{-/-}$ mutant embryos, otic vesicles are smaller and patterning is affected without major defects in lumen formation [64,65]. Different patterning defects are found in *fgf3*$^{-/-}$ mutants [63,66], but again, lumen formation appears to be normal, suggesting that in zebrafish, Fgf signalling could be dispensable or dependent on other Fgfs. Later on, expansion of the lumen is mediated partly by the ingression of fluid coming from otic epithelial cells, which shrink, remodel and actively participate in the process of lumen growth [38]. In chick, changes in cellular volume during the expansion phase of the otic vesicle have not been quantified, but dorsal otic epithelial cells undergo rapid thinning [67,68] (see also below). An exciting possibility is that dorsal thinning is accompanied by fluid pumping into the lumen, as happens during the early stages of lumen formation in zebrafish [38].

In the zebrafish lateral line primordium, an early step in the morphogenesis of sensory epithelia (future neuromasts) is the formation of rosettes through apical constriction of polarised cells. Through the use of live imaging combined with genetic and pharmacological manipulations, rosette formation was found to be dependent on Fgfr-Ras-MAPK signalling, leading to activation of Rho-associated kinase (Rock2a) and Myosin II [69]. Shroom3, which is a target of Fgf signalling, is also required for rosette formation [70]. It will be interesting to learn whether the initial lumens that form at the poles of the zebrafish otic vesicle share properties with the microlumens described for the lateral line, which act to concentrate secreted Fgf, ensuring the co-ordinated response of cells within a rosette that share a lumen [71].

In species where the otic placode invaginates and pinches off from the overlying ectoderm, vesicle closure correlates with a focus of increased tissue apoptosis [68], but whether blockade of cell death prevents closure has not been directly tested. In chick, the position of pinching off seems to correlate with a medio-lateral cell lineage and gene expression boundary (medial: *Pax2*; lateral: *Soho1*) [72]. *Pax2* mutant mice, while displaying gross morphogenetic defects in cochlear and semicircular canal growth, have normal otic vesicle closure, but interestingly, in *Pax2;Pax8* double null mice, there is defective invagination of the otic cup, which remains continuous with the ectoderm [73]. Here again, Pax2/8 transcription factors emerge as good candidates to couple patterning with morphogenesis.

3. Formation of the VIIIth ganglion: delamination and migration of neuroblasts

Statoacoustic ganglion (SAG) neurons of the eighth (VIIIth) cranial ganglion are responsible for the transmission of vestibular and acoustic sensory information to the brain. The SAG, initially located anteroventral to the otic vesicle, contains the cell bodies of bipolar afferent neurons that project on the one side to hair cells embedded in the otic epithelium and on the other to the acoustic or vestibular nuclei of the brainstem. Unlike those in other cranial sensory ganglia, SAG neurons are thought to have their origin in the otic placode, with very little contribution from the neural crest [1,74]. Formation of the SAG is a sequential process; firstly, otic neuronal precursors are specified within the otic epithelium by the proneural gene, *neurog1*, and then delaminate as neuroblasts after activation of another proneural gene, *NeuroD* [75]. Once they have left the

otic epithelium, neuroblasts continue proliferating to expand the neuronal population and finally differentiate within the SAG [1,76]. Details of the specification of the neurogenic domain and progression of otic neurogenesis have been well characterised and are reviewed elsewhere [76–79]. Here we focus on the cellular events leading to the delamination and migration of otic neuroblasts.

Several experiments reveal that vestibular and auditory neurons of the SAG derive from distinct populations of neuronal precursors within the neurogenic domain and also arise sequentially; early born neuroblasts constitute the vestibular ganglion and later-born neurons the auditory portion of the SAG [80,81]. Genetic tracing of *Neurog1*-positive cells in the mouse was performed at distinct temporal windows; when neuronal precursors were labelled at E8.5 they generated almost exclusively vestibular neurons whereas auditory neurons derived from neuronal precursors labelled at E12.5 [81]. In chick, similar results were found when analysing clones derived from retrovirus injections or from spatially restricted DiI/DiO injections [82,83]. Moreover, in these studies and in the work of Raft and colleagues, a clonal relationship between sensory neurons and hair cells from the utricular macula was also revealed [84].

In chick, delamination occurs over a prolonged period spanning from otic cup and late otic vesicle stages (E6), with a peak of delamination at stages 16–17 [1,83,85]; in zebrafish, otic neurogenesis takes place from 17 h post fertilisation (hpf) until 42 hpf [53,86]. Unfortunately, the information regarding the dynamics of cellular behaviours leading to the delamination and migration of otic neuroblasts is still scarce. It is debated whether neuroblasts delaminate from placodes by a mechanism of epithelial-mesenchymal transition (EMT) as shown for neural crest cells exiting the dorsal neural tube. Hallmarks of EMT of cranial neural crest are a set of transcriptional profiles that lead to specific cellular morphological changes, including a switch from an epithelial to mesenchymal cellular phenotype and migratory properties reviewed in [87–90]. Briefly, in chick, Bmp4 and Wnt1 trigger the expression of the transcription factor genes *Snail/Slug*, *Foxd3*, *Sox9* and *Sox10*, the products of which co-operatively induce a switch in expression of adhesion molecules (N-cadherin to cadherin6B, 7 and 11) and activation of RhoB. At the epithelial level, the basal lamina is degraded; neural crest cells lose apicobasal polarity, translocate basally and acquire a mesenchymal phenotype as they exit [88]. When some of these features were analysed for delaminating neuroblasts of diverse placodes in the chick, however, neither expression of *Snail2*, activation of *RhoB* nor a mesenchymal phenotype was observed [91]. Altogether, it was concluded that sensory neuroblasts do not delaminate by an EMT mechanism. However, it has become evident in recent years that EMT is not an all-or-nothing event and intermediate types of EMT are present during development [89]. Moreover, in chick, other transcription factors such as *Twist*, *Zeb* and *E47* have been implicated in EMT and thus could be acting instead of *Snail/Slug* [90]. Finally, *Snail* expression is absent in chick placodes but present in the zebrafish otic vesicle during delamination [65,92], reinforcing the idea that further work should be done to analyse the parallels between delamination of otic neuroblasts and neural crest cells.

After neuroblasts have emigrated from the epithelium, they coalesce into a highly packed globular mass of cells. The most proximal domain of the SAG (closer to the epithelium) contains mitotically active, *NeuroD*-positive neuroblasts, while the distal portion of the SAG contains earlier-born neurons in which division has ceased. This distal population already expresses neuronal differentiation markers such as neurofilaments, neurotrophin receptors or Islet1 [76,83,86]. Waves of FGF signalling, mediated by different FGF ligands, regulate the process of neurogenesis and SAG maturation [86,93–95]. It has been proposed that levels of FGF signalling dictate the outcome of neurogenesis. Initially, low activity of FGF signalling promotes neuroblast emergence in the neurogenic domain, but later on, increased levels of FGF signalling by *fgf5* expressed in SAG neuroblasts feed back onto the neurogenic epithelium to terminate neuroblast specification [86]. Interestingly, conditional manipulation of FGF signalling in the zebrafish (using heat shock-inducible transgenes and pharmacological interference) implicates Fgfr/PI3K/Akt signalling in zebrafish otic neurogenesis, and Fgfr/Erk1/2 signalling in hair cell production [95]. Other signals such as NGF and Igf-1 have also been implicated in SAG growth [96–98]. But how does communication between otic neurons take place to elicit proliferative, migratory and structural changes within the SAG? Cytonemes, long thin filamentous cellular protrusions, have recently been discovered as communication bridges between cells [99]. Growth factors are transported along cytonemes and delivered very precisely to neighbouring cellular membranes [100,101]. Whether SAG neurons use cytonemes to regulate their physiology or migration is interesting but still unexplored. Another untested possibility is that as neuroblasts exit the epithelium, they use forces to push the previously-born neurons forward, making the later stages of neuronal migration mainly mechanical and passive. Direct visualisation of otic neurons will elucidate the nature of neuronal migratory movements within the SAG or other cellular behaviours not conceived of from observations in fixed tissue.

Another influence on otic neuroblast migration is likely to be the neural crest, although its importance appears to be dependent on species or placodal type. In the chick, the neural crest establishes a corridor for epibranchial-derived neuroblasts during their migration; physical separation of the neuroblasts from the mesoderm by these neural crest corridors has been suggested to be essential for their correct development and axonal growth to the central nervous system [102]. Similarly, blocking the formation of neural crest cell precursors in the zebrafish with leflunomide results in SAG disorganization and axonal branching defects [103]. In the mouse, however, a loss of Schwann cells through conditional knockout of *Sox10* in the neural crest disrupted peripheral innervation of the cochlea by spiral ganglion neurons, but their central projections to cochlear nuclei were unaffected [104].

4. Segregation of sensory epithelia and morphogenesis of sensory chambers

In most organisms, differentiation of sensory hair cells occurs after otic neurogenesis, although the processes are concomitant in the zebrafish. In all species, however, formation of the otic sensory patches begins with a relative thickening of ventral epithelium that is accompanied by a progressive thinning of dorsal epithelium. Cells in dorsolateral regions adopt a thin squamous morphology; these are destined to form non-sensory derivatives, including the semicircular canal ducts (described in more detail below) and endolymphatic duct. The thickened ventral prosensory region, on the other hand, gives rise to the various sensory epithelia of the ear, which differentiate into two main cell types: sensory hair cells, which sit in an apical position, and supporting cells, which span the apical-to-basal width of the epithelium and have their nuclei positioned basally (Fig. 2).

Different vertebrates have different numbers of sensory patches (maculae, papillae and cristae) in the ear—six or seven in mammals, seven in the zebrafish, eight in the chicken and nine in some species of limbless amphibian [105–107]. The formation of multiple distinct sensory domains during development is thought, in part, to reflect the separation of initially contiguous prosensory regions, observed well over a hundred years ago [108], as reviewed in [106,109]. This is supported by expression pattern data from different species, in which genes such as *Lfng*, *NT3* and *Fgf10* initially mark broad prosensory (or 'sensory-competent') domains

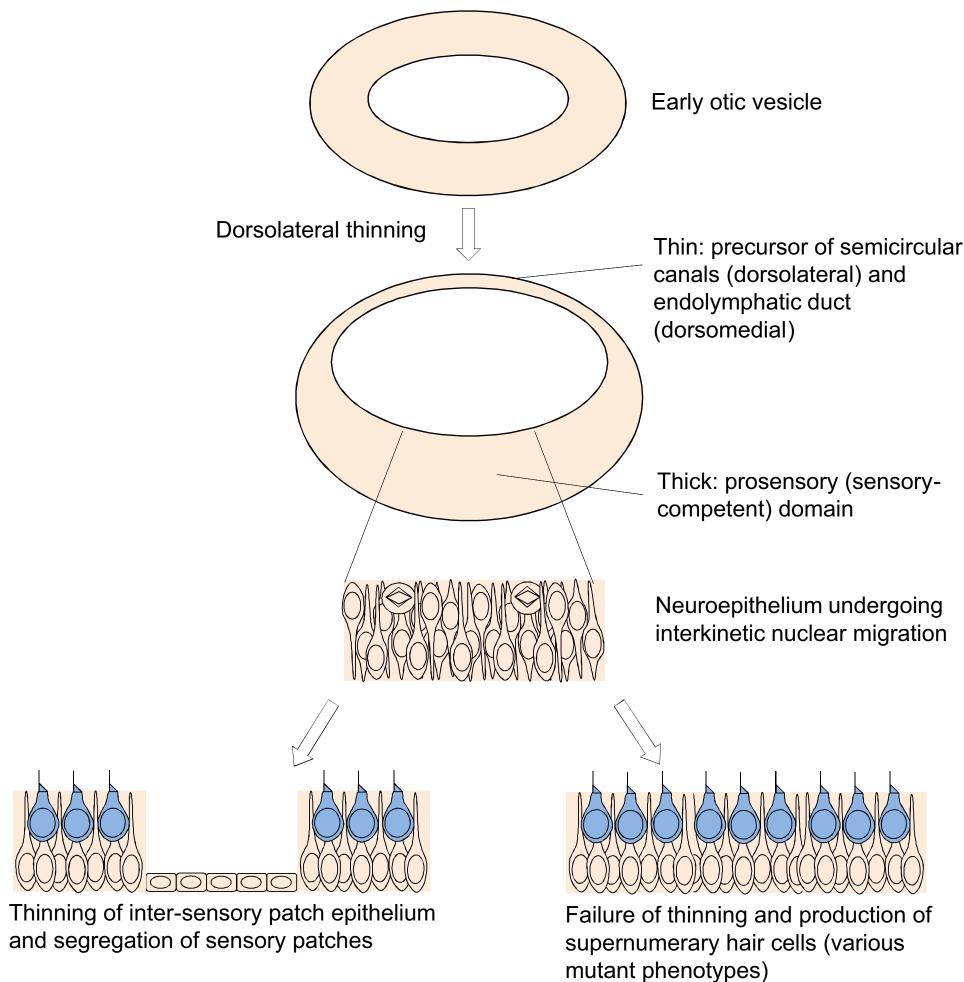

Fig. 2. Generation and segregation of sensory epithelia in the ear. Top: schematic diagram of a generalised otic vesicle (lateral view). Soon after hollowing or invagination, there is a relative thinning of dorsal epithelium (dependent on BMP signalling in the chick), whereas ventral otic epithelium contains regions of thickened prosensory neuroepithelium undergoing interkinetic nuclear migration. To generate individual and separate sensory patches, some regions become thin, whereas others give rise to differentiated sensory hair cells (blue) (lower left). In the zebrafish, thinning correlates with expression of E-cadherin. Mutation of *Lmx1a*, *Foxg1* (mouse), *fgf3* or *jag1b* (zebrafish) can result in a failure of epithelial thinning, fusion of sensory epithelia and, in some cases, production of supernumerary hair cells. Based on information from references cited in the text.

that are later subdivided into discrete sensory patches [110–113]. In zebrafish, a model has been proposed in which Notch signalling is required to separate the initial prosensory equivalence group into two domains, prefiguring the utricular and saccular maculae [114]. Nevertheless, this cannot be the sole mechanism, as supernumerary hair cells still form in two discrete clusters in the *mindbomb* mutant ear, in which Notch signalling is disrupted [115]. Ectopic hair cells can differentiate across the entire prosensory region, however, in zebrafish embryos treated with retinoic acid or with reduced Fgf signalling from the 18–20 somite stage [66].

Further genetic evidence for the mechanisms underlying the segregation of sensory epithelia comes from analysis of mutant phenotypes in which sensory regions remain undivided, although the details are far from understood. In mice and zebrafish, maculae are fused or incompletely separated in *Fgf3, Hmx3, Lmx1a*, N-*myc, Otx1, Otx2* and *Tbx1* mutants or morphants [63,66,116–124]. The loss of intervening non-sensory tissue between the sensory domains in these mutants can correlate with expression of the relevant gene: expression of the LIM-homeodomain transcription factor gene *Lmx1a*, for example, becomes progressively restricted to non-sensory regions, where it is thought to limit signalling within or between sensory epithelia [120]. Separation of the anterior and lateral cristae from a single domain is dependent on *Foxg1* in the mouse: *Foxg1*$^{-/-}$ mutants frequently have a single ampulla shared by both anterior and lateral canal ducts, which can contain a fused crista [125,126]. It will be interesting to explore whether the downstream targets of patterning genes such as *Lmx1a* and *Foxg1* include genes coding for cytoskeletal or cell adhesion proteins, which could mediate the cellular remodelling events required to convert the pseudostratified prosensory epithelium into a squamous non-sensory epithelium.

In the zebrafish, FGF signalling is emerging as a key player in driving the separation of sensory domains. In the *fgf3*$^{-/-}$ mutant, the utricular and saccular maculae remain undivided, and supernumerary hair cells form in the saccular macula [63,66]. Additional FGF ligands appear to be required for correct formation of the cristae. In the *jag1b*$^{-/-}$ mutant, both anterior and posterior cristae are lost, apparently through different FGF-dependent mechanisms [127]. In this study, Ma and Zhang propose that Fgf10a acts as a survival signal for posterior crista sensory tissue; posterior *fgf10a* expression and the posterior crista are lost in the *jag1b* mutant ear. By contrast, a zone of FGF/ERK signalling, possibly mediated through Fgf8a, is extended in the anterior prosensory domain of the *jag1b* mutant ear. This correlates with extension of a zone of flattened, E-cadherin-positive cells that normally separates the anterior and lateral cristae, resulting in loss of the anterior crista.

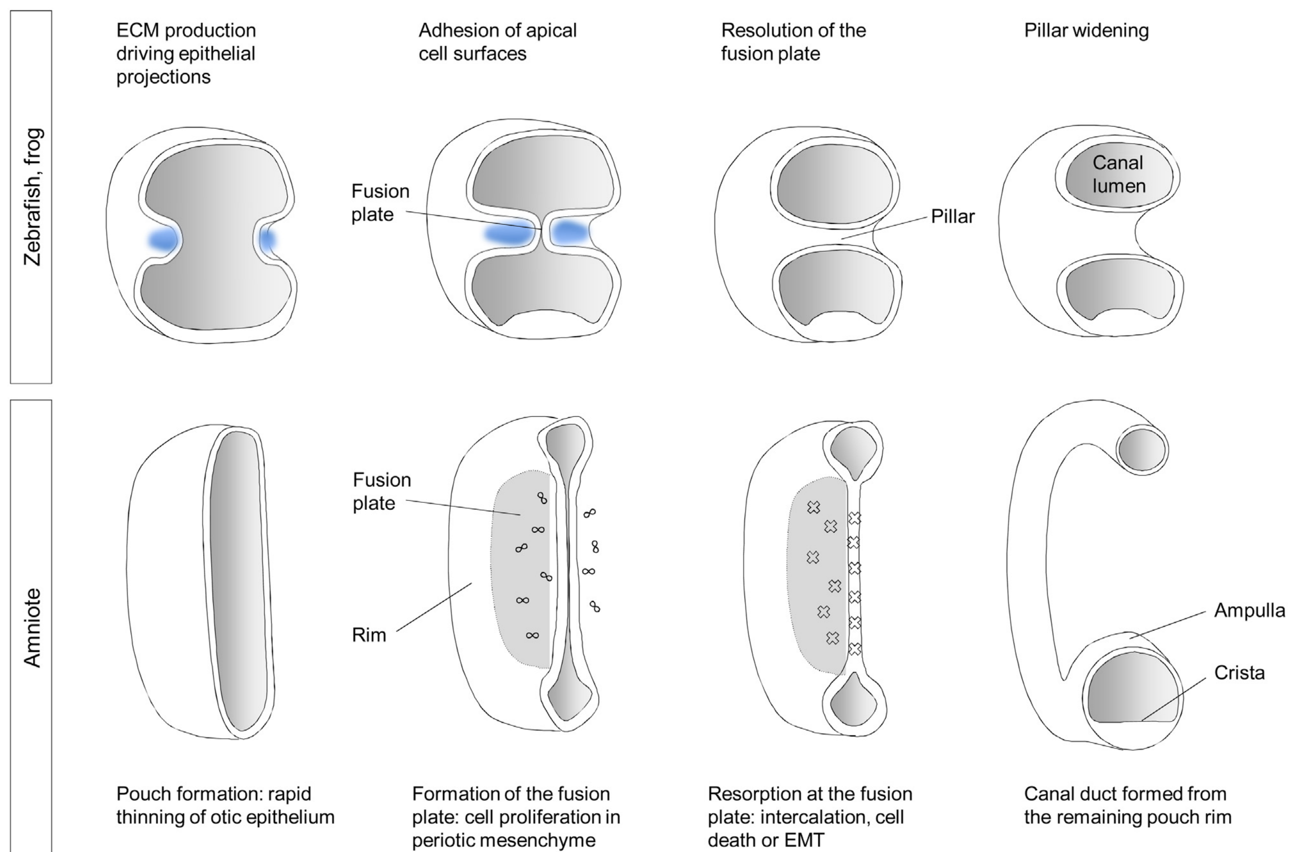

Fig. 3. Simplified schematic diagram to compare semicircular canal formation in the zebrafish or frog ear with that in amniotes. Only one canal is illustrated; not to scale. In fish and frog (top row), the production of extracellular matrix (blue) helps to drive epithelial projections into the otic lumen. In amniotes (bottom row), a canal pouch forms by thinning of the otic epithelium. Cell division in surrounding mesenchyme (shown as ∞∞) pushes the sides of the pouch together. In all species, cells adhere, fuse and resolve at a fusion plate. In the frog and fish, this leaves a pillar of tissue spanning the otic lumen; in the chick, cells are cleared in this area by apoptosis (shown as xx). Cell clearance leaves the canal duct formed from the remaining rim of epithelium. Further widening of the pillar, canal growth and formation of the ampulla and crista lead to the mature semicircular canal.

Conversely, in embryos treated with pharmacological inhibitors of FGF signalling or ERK phosphorylation, the anterior and lateral cristae remain as a single undivided zone of thickened epithelium containing supernumerary hair cells [127]. This phenotype differs from that of the *fgf3* mutant, where cristae appear relatively normal [63], reinforcing the conclusion that different FGF ligands play quite distinct roles in otic patterning and morphogenesis in the zebrafish ear.

In the mature ear, the sensory epithelia are not only segregated from one another, but some are also partitioned into distinct sensory chambers or recesses, connected to the rest of the ear by narrower foramina. In particular, the utriculosaccular foramen constricts to form a very narrow link joining the inferior and superior parts of the ear (reviewed in [128,129]). Epithelial constriction to form the separate recesses is dependent on the function of *Otx1* [121], *Lmx1a* [120] and *N-myc* [123], and correlates with zones of increased cell death [130]. As the connections between the chambers narrow, so the chambers themselves expand in size. In amniotes, the cochlear duct elongates via cell proliferation, cell–cell intercalation and convergent extension; in mammals, the duct also coils into the shell-like spiral that gives the cochlea its name (reviewed in [131,132]). Pharmacological inhibition experiments using cultured explants of cochlear epithelium have demonstrated an autonomous role for myosin II in driving convergent extension movements in the mouse cochlea [133]. The planar cell polarity (PCP) pathway, mediated by non-canonical Wnt signalling, is also an important contributor to cochlear morphogenesis. Conditional mutation of *p120-catenin* has been shown to uncouple the downstream effectors of PCP that mediate convergent extension and hair cell polarity in the mouse cochlea [134]. This study also charts the dynamic changes in cellular contacts and cadherin expression that occur during development of the cochlea [134]. Another recent study highlights the role of Fgf10 in cochlear duct morphogenesis. As in the vestibular system, *Fgf10* is expressed in cochlear sensory epithelium, whereas expression of its receptor *FgfR2B* is in domains of non-sensory tissue. *Fgf10*$^{-/-}$ mutants have a shorter and narrower cochlear duct that lacks non-sensory derivatives (Reissner's membrane and the outer sulcus) [135].

5. Morphogenesis of the semicircular canals

The semicircular canal system detects rotational movements (angular accelerations) of the head; its primary function, effected via reflexive eye and neck movements, is to stabilise gaze during motion. Three semicircular canals, arranged orthogonally to one another, are present in the inner ears of all gnathostome (jawed) vertebrates, but their relative size to the rest of the ear and body differs widely between species. Fast-moving arboreal mammals, which perform agile leaps with great accuracy, have long, thin semicircular canal ducts for maximal sensitivity, whereas slow-moving mammals—together with whales and dolphins, which are acrobatic but have limited neck motility—have relatively small semicircular canals [136,137]. Cartilaginous and bony fish display a wide variety of semicircular canal morphologies [138,139]. Interestingly, the origins of upright stature and bipedalism in humans can be traced through the evolution of semicircular canal morphology [140]. The

mechanisms underlying the generation of these species-specific differences are little known; however, studies of otic abnormalities in the mouse stretch back for over fifty years, and some of the fundamental steps of semicircular canal morphogenesis in this species are now reasonably well understood.

In amniote ears, the first step of semicircular canal formation is the appearance of two pouches or diverticula in the otic vesicle—a dorsal pouch that will give rise to the anterior and posterior canals and crus commune, and a lateral pouch that prefigures the lateral (horizontal) canal. Pouch formation and growth are driven by rapid thinning of the dorsolateral otocyst epithelium, rather than a local increase in cell proliferation [68]. In the chick, this thinning involves transition from a columnar to a squamous cell shape, a process that is dependent on BMP signalling, and correlates with changes in the distribution of E-cadherin [67]. To form the semicircular canal ducts from the pouches, the pouch sides move towards each other and fuse: cell clearance at the fusion plate in the centre of the pouch leaves the semicircular canal duct, which develops from the remaining pouch rim (Fig. 3). In the mouse ear, the first step in this process involves a loss of epithelial morphology in the pouch sides, with concomitant disruption of the underlying basement membrane [141]. It is thought that cell proliferation in periotic mesenchyme contributes to the forces pushing the sides of the fusion plate together; in both *Netrin1* and *Fgf9* mutants, the mitotic index is reduced in surrounding mesenchyme, and fusion plates fail to form [142,143]. In the zebrafish or *Xenopus* ear, formation of pouches is less obvious in the small and compact otic vesicle. Here, finger-like projections of epithelium grow towards each other, driven not by mesenchymal proliferation, but by production of extracellular matrix [144–146]. Three such projections meet with corresponding bulges from a lateral projection, where they fuse to form three pillars of epithelium spanning the otic lumen.

In all species, cells at the fusion plate must recognise each other, touch, and fuse or intercalate. The proteins required for cell adhesion at the fusion plate have not yet been identified, but one candidate in zebrafish is the adhesion class G protein-coupled receptor Gpr126 [145]. The murine orthologue is expressed in the mouse ear [147], although it is not yet known whether it performs a similar role as in zebrafish. In the zebrafish mutant, epithelial projections within the ear overgrow and fail to down-regulate the expression of genes coding for a variety of extracellular matrix components. Cell adhesion and rearrangement at the fusion plate fails, and thus pillars and canal ducts are unable to form in the *gpr126* mutant ear [145].

Once the fusion plate has formed in the wild-type ear, cells are cleared from this area, leaving the canal duct formed from the surrounding rim of the pouch. In the chick, apoptosis is thought to be a major contributor to cell clearance [148], whereas in the mouse, some fusion plate cells are resorbed back into the duct epithelium [141]. It is also possible that other cells undergo an EMT and become part of the periotic mesenchyme. In the zebrafish, where the fusion plates are much smaller than those in the amniote ear, cell death does not appear to be a major player [146,149], but the destination of fusion plate cells has not yet been traced in detail.

Correct formation of the canal duct from the pouch depends on a fine balance of cell behaviours at the fusion plate: too much cell clearance, and the canals will be thin or truncated; too little, and the result is an unfused canal pouch, remaining as an undivided vesicular structure (reviewed in [150]). In the mouse ear, the extent of cell clearance at the fusion plate correlates with the domain of *Netrin1* expression, and is dependent on cross-inhibitory interactions with *Lrig3* to form the lateral semicircular canal duct [151] and with *Dlx5* to form the anterior and posterior semicircular canals [152]. Maintenance of *Dlx5* expression in the rims of the anterior and posterior canals (and thus protection from Netrin1-dependent resorption) is dependent on a cascade of sensory-dependent Wnt, Bmp and Fgf signalling. Mosaic depletion of β−catenin in fusion plate cells has also revealed a second, later role for Wnt signalling in mediating resorption at the fusion plate [152].

One hypothesis that has attracted much attention over the last decade posits that formation of the non-sensory canal ducts is dependent on signalling from the developing sensory cristae to establish a 'canal genesis zone' [131,153]. This idea is supported by evidence from various mutant phenotypes: while ampullae and cristae can form in the absence of canal ducts, examples of normal canal ducts without sensory cristae are rare, and in many mutants, both sensory and non-sensory tissues are affected together. Candidate signalling molecules that are expressed in the sensory domains include those of the Bmp, Fgf and Wnt families, where one of their roles is to maintain *Dlx5* expression in the canal rims, as described above. *Bmp4*, which is expressed in the cristae in several vertebrate species, is required for the development of both cristae and canal tissue in the mouse ear [154]. In turn, *Bmp4* is required for the normal expression of *Bmp2b*; conditional loss of *bmp2b* function in the zebrafish results in the loss of all three canal ducts, but cristae develop relatively normally [155]. *Fgf10* also makes an interesting case study: it is expressed in the cristae, whereas the gene coding for its receptor, *FgfR2(IIIb)*, is expressed in non-sensory epithelium [156]. The posterior canal is most severely affected in murine *Fgf10* mutants: both the canal and its crista are missing in homozygous mutants, whereas heterozygous mutants reveal a dose dependency for *Fgf10*, with a smaller or absent posterior semicircular canal [135,157,158]. Retinoic acid (RA) signalling is also likely to be involved in semicircular canal morphogenesis; in the mouse, mutants for the RA-synthesising enzyme gene *Raldh3* have small and thin semicircular canals [159].

Not all mutant phenotypes support the canal genesis zone model, however. Mice mutant for *Jag1* or *Sox2*, for example, have ears that lack one or more ampullae and cristae, but the crus commune and canal ducts (although truncated) are present [160–163]. Thus the canal genesis zone model, whilst providing a useful framework for understanding vestibular morphogenesis, cannot account for all mutant phenotypes, and data from the *Sox2* and *Jag1* mutants argue for a degree of independence from sensory signalling in the development of non-sensory elements of the semicircular canal system.

In addition to *Dlx5* [164,165], mutations in several other transcription factor genes result in semicircular canal defects. These genes include *Gbx2* [166], *Hmx3* (previously *Nkx5.1*) [167–169], *Lmx1a* [120,170] and *Prx1* and *Prx2* [171]. Homozygous loss of function of the *Chd7* gene, which codes for a chromatin remodelling enzyme, results in reduced or loss of expression of several genes involved in semicircular canal formation, including *Hmx3* and *Otx1* (see below); as a result, semicircular canals are lost altogether in $Chd7^{-/-}$ mutant ears [172]. Interestingly, lateral canal defects in $Cdh7^{+/-}$ mice can be rescued by treatment with an inhibitor of Retinoic Acid (RA) synthesis [173].

The lateral (horizontal) semicircular canal is the last to form during embryogenesis, and is also thought to have been the last to evolve. Interestingly, in a study of human inner ear structural anomalies, the lateral canal was been found to be the most commonly affected [174]. One essential and highly conserved factor for the formation of the lateral canal is the homeodomain transcription factor Otx1. Otic expression of *Otx1* mRNA correlates with the presence of a lateral horizontal canal in several species [175], whereas the lamprey, which lacks a lateral horizontal canal, lacks an otic domain of *Otx1* expression [176,177]. The lamprey ear, instead, has paired medial canals that mediate sensitivity to movement in the horizontal plane [178]. In both mouse and zebrafish, the loss of *Otx1* function results in loss of the lateral canal and crista [116–118,121]. Loss of the lateral canal has also been attributed to

Table 1
Semicircular canal mutant phenotypes in the mouse and zebrafish.

Main phenotype[a]	Mutated gene(s)[b]	Species	Reference
All three canal ducts, ampullae and cristae variably affected or missing	*Bmp4*	Mouse	[154]
	Cdh7	Mouse	[172]
	Dlx5, Dlx5;Dlx6	Mouse	[164,165,188]
	Hmx2, Hmx3	Mouse	[167–169]
	Wnt1;Wnt3a	Mouse	[189]
	Zic2	Mouse	[190]
All three canal ducts missing; ampullae and cristae present	*bmp2b*	Zebrafish	[155]
Partial development of canal ducts in the absence of one or more ampullae and cristae	*Jag1*	Mouse	[161–163]
	Sox2	Mouse	[160]
Anterior and posterior canals and crus commune truncated or missing	*Mafb*	Mouse	[191]
	Gbx2	Mouse	[166]
Lateral canal small, truncated or missing	*Gli3*	Mouse	[180]
	N-myc	Mouse	[123,124]
	Otx1	Mouse and zebrafish	[116–118,121]
	Prx1/2	Mouse	[171]
	Shh	Mouse	[179,180]
Anterior canal small or truncated	*Casp3*	Mouse	[192] – lateral canal function also affected
Posterior canal small, truncated or missing	*Fgf10*	Mouse	[135,157,158]
	Six1/Eya1 compound heterozygotes	Mouse	[193]
Failure or delay in fusion or cell clearance at the fusion plate	*cβcat* (ClassC and constitutively active allele)	Mouse	[152]
	Cdh7 heterozygotes	Mouse	[172]
	Fgf9	Mouse	[143]
	gpr126	Zebrafish	[145]
	Lmx1a	Mouse	[120,170]
	Netrin1	Mouse	[142]
Excess cell clearance at the fusion plate; thin, truncated or missing canals	*cβcat* (Class A)	Mouse	[152]
	Lrig3 (lateral canal)	Mouse	[151]
Thin, irregular, or discontinuous canals	*Alk3-CKO; Alk6+/–*	Mouse	[194]
	Nor1	Mouse	[195] Mutants have flattened ampullae
	Raldh3	Mouse	[159]
	Zeb1	Mouse	[196]
Missing, rudimentary or thin projections or pillars (zebrafish)	*hdac1*	Zebrafish	[197]
	ptc1+/–;ptc2-/-	Zebrafish	[198]
	sox10	Zebrafish	[199]
	tbx1	Zebrafish	[181]
	ugdh	Zebrafish	[200]
Overgrown projections (zebrafish)	*dzip1, hip1, ptc2* (ventral projection only)	Zebrafish	[198]
	gpr126	Zebrafish	[145]

[a] Phenotypes are often variable; the main defects are described here, but there are often classes of differing severity, or additional defects may be present.
[b] Most mutations described are homozygous loss-of-function, but some studies describe heterozygous phenotypes or different allelic variants. See the individual references for details.

the altered expression of *Otx1* in the ears of murine *Shh*$^{-/-}$ mutants [179,180]. Regulation of the size of the *otx1b* expression domain in the zebrafish otic vesicle appears to be dependent on the opposing activities of Fgf and Retinoic Acid (RA) signalling: Fgf promotes, whereas RA restricts, otic *otx1b* expression [66]. Otic *otx1b* expression is also lost in zebrafish *tbx1* mutants [181] and *sparc* morphants [182].

Although many genes required for semicircular canal formation have been identified, the challenge is now to link signalling pathways and transcription factor activity to specific cell behaviours, in order to generate a unified model of canal formation in the ear. By grouping together similar mutant phenotypes (Table 1), and comparing to data from the frog and chick, it may be possible to infer links between different gene products that can then be tested experimentally. A recent study using loss- and gain-of-function approaches in the chick implicates both canonical and non-canonical BMP signalling in the regulation of *Dlx5* and *Hmx3* expression in the dorsal otocyst [183]. New candidate genes with roles in otic morphogenesis are being identified through analysis of insertional mutants in the mouse [184,185] and mutagenesis screens in *Xenopus tropicalis* [186]. There are some notable gaps in our understanding of semicircular canal formation: in particular, very little is known about formation of the dividing septa that delineate the canal ducts in the zebrafish, or of the ampullae at the base of each duct that house the cristae, both of which require the generation of zones of high epithelial curvature. It will be interesting to test whether this requires apoptosis and myosin II-dependent pulling forces, as has been shown during epithelial folding in *Drosophila* [187].

6. Biomechanics and live imaging: converging approaches to understand morphogenesis

Most of the attention and knowledge so far on how organs develop has focused on the genetic basis of cell identity, communication and tissue patterning due to the feasibility of studying gene activity and its manipulation in whole embryos. The morphological changes underlying organogenesis have also been taken into account, but with limited resolution of the spatiotemporal dynamics and consideration of the biophysical properties of tissues until recently. The advent of powerful non-invasive live-cell imaging and labelling techniques is revolutionising the developmental biology field. In particular, light-sheet microscopy now enables long time-lapse imaging deep in tissues, in all spatial dimensions, with high temporal resolution and little phototoxicity [201–203], providing unprecedented new information on the kinetics, dynamics, and

3D architecture of morphogenesis at the cellular, tissue or organ level. In addition, the physics of morphogenesis is beginning to be elucidated by combining visualisation of fine-grained sub-cellular details with novel non-invasive nano/picoscale technologies for mechanical manipulation of tissues. Some of the tools for monitoring mechanics forces are laser-cutting devices, micropipettes to analyse mechanical and adhesive properties of cells and tissues, and, finally, molecular force sensors. A plethora of relevant data on the impact of forces, tensions, pressure and flows in embryogenesis has been published recently focusing on gastrulation, heart and endothelial development, among many others (see [204–206]).

The inner ear, as a highly 3D sophisticated organ that undergoes extensive tissue remodelling during development, constitutes an excellent model to tackle the question of how biomechanics, tissue morphogenesis and gene regulation are coupled. Have the advancements described above impacted on our understanding of inner ear development? To date, most live imaging data concerning inner ear development has focused on preplacodal movements or placode formation dynamics, due to their accessibility [20,22,23]. To our knowledge, laser microsurgery experiments to quantify mechanical forces in the inner ear have only been reported during lumen formation in the zebrafish inner ear [38]. There, laser cuts of the apical membrane in mitotically rounded cells revealed a mechanical role exerted by those cells over the luminal membrane to expand the lumen. Further work in this direction is needed to link the cellular events with the physical properties of tissues during otic morphogenesis. In addition, light-sheet microscopy promises to provide very interesting dynamical data on morphogenetic processes beyond placode stages—such as cochlear extension and coiling, semicircular canal duct formation or hair cell positioning—in the near future. The application of these imaging and physical techniques will also mean that mutant phenotypes can be explored in new ways, leading to a deeper understanding of inner ear morphogenesis at a systems level.

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