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Cells, molecules and morphogenesis: The making of the vertebrate ear

Bernd Fritzsch^{*}, Sarah Pauley, and Kirk W. Beisel

Creighton University, Department of Biomedical Sciences, 2500 California Plaza, Omaha, NE 68178, USA

Abstract

The development and evolution of mechanosensory cells and the vertebrate ear is reviewed with an emphasis on delineating the cellular, molecular and developmental basis of these changes. Outgroup comparisons suggests that mechanosensory cells are ancient features of multicellular organisms. Molecular evidence suggests that key genes involved in mechanosensory cell function and development are also conserved among metazoans. The divergent morphology of mechanosensory cells across phyla is interpreted here as 'deep molecular homology' that was in parallel shaped into different forms in each lineage. The vertebrate mechanosensory hair cell and its associated neuron are interpreted as uniquely derived features of vertebrates. It is proposed that the vertebrate otic placode presents a unique embryonic adaptation in which the diffusely distributed ancestral mechanosensory cells became concentrated to generate a large neurosensory precursor population. Morphogenesis of the inner ear is reviewed and shown to depend on genes expressed in and around the hindbrain that interact with the otic placode to define boundaries and polarities. These patterning genes affect downstream genes needed to maintain proliferation and to execute ear morphogenesis. We propose that fibroblast growth factors (FGFs) and their receptors (FGFRs) are a crucial central node to translate patterning into the complex morphology of the vertebrate ear. Unfortunately, the *FGF* and *FGFR* genes have not been fully analyzed in the many mutants with morphogenetic ear defects described thus far. Likewise, little information exists on the ear histogenesis and neurogenesis in many mutants. Nevertheless, a molecular mechanism is now emerging for the formation of the horizontal canal, an evolutionary novelty of the gnathostome ear. The existing general module mediating vertical canal growth and morphogenesis was modified by two sets of new genes: one set responsible for horizontal canal morphogenesis and another set for neurosensory formation of the horizontal crista and associated sensory neurons. The dramatic progress in deciphering the molecular basis of ear morphogenesis offers grounds for optimism for translational research toward intervention in human morphogenetic defects of the ear.

Keywords

Hair cell; Secondary sensory cell; Neuron; Atoh1; Neurog1; Ear evolution

1. Introduction

Sensation of mechanical, chemical and photic stimulation is found in protists and thus predates multicellular organisms (Metazoa). Molecules and organelles to perceive such stimuli therefore likely evolved prior to the formation of Metazoa (Gehring, 2005). With the

evolution of metazoans, individual sensory cells became specialized to enhance sensory communication with their environment. Such specialized cells eventually evolved from single cells to multicellular organs. These arrays of sensory cells enabled animals to effectively orient, for example, in gravity. Extant examples of single mechanosensory cells are found in all animal phyla (Markl, 1974) and formation of mechanosensory organs that allowed perception of stimuli in a directed fashion is known in all diploblastic phyla (Arkett et al., 1988; Jorgensen, 1989; Markl, 1974). These organs perceive either gravity-mediated linear acceleration (statocysts), or water movement across the organ surface (hydrodynamic receptors). The evolution of such gravity-sensing sensory systems was accompanied by the formation of calcium precipitates in an extracellular matrix tethered to a detector that allowed gravistatic orientation in space.

One aspect almost unique to the ear is the intertwining of form and function as reception of specific physical sensations requires differential stimulation of specialized sensory epithelia. This need for a precise geometry of the inner ear adds another level of complexity: A three dimensional structure must be generated and mechanosensory cells must be specifically placed and covered with a specific extracellular matrix to permit extraction of only certain mechanical stimuli. Although our understanding of this interactive process is limited, data indicate that this combined morphogenetic and histogenetic process is tied into a global patterning process of the ear (Bok et al., 2005; Chang et al., 2004a; Fritzscht and Beisel, 2004; Lin et al., 2005). In contrast, our knowledge of the molecular basis necessary to form the three dimensional structure of the ear has dramatically advanced within the last few years, owing to investigations of numerous null mutant lines (Bober et al., 2003; Chang et al., 2004b; Fekete and Wu, 2002; Pauley et al., 2003; Pirvola et al., 2000, 2004; Riccomagno et al., 2002, 2005; Wang and Lufkin, 2005; Wright and Mansour, 2003b).

The purpose of this review is to (i) describe the cellular and molecular basis of mechanosensory cell evolution; (ii) present an overview of the evolution of multicellular statocysts and the vertebrate ear; and (iii) present an overview of molecular interactions necessary for ear morphogenesis, focusing in particular on the semicircular canals. We will, wherever possible, attempt to specify the developmental alterations necessary to make known evolutionary changes in ear morphology.

Outgroup comparisons (i.e., compare craniate with non-craniate chordates) using cladistic analysis to distinguish likely ancestral (plesiomorphic) characters from derived (apomorphic) ones will be performed to establish character evolution. We want to emphasize that in addition to the classical morphological concept of 'homology' (i.e., similarities derived from the same ancestral structure) we will rely on molecular data to establish 'sameness' of dissimilar looking cells and organs. The latter approach will accommodate the increasingly available genetic information which has extended the concept of 'homology' from structure to molecules. Such concepts are referred to as 'deep homology' (Shubin et al., 1997) or 'homocracy' (Nielsen and Martinez, 2003).

It needs to be stressed that although similarities in molecular or developmental regulatory modules do not necessarily demonstrate 'homology', unique constellations of co-expressed genes allow raising such questions of 'deep homology' or 'homocracy' for otherwise dissimilar looking cells or organs. Nevertheless, these issues are controversial and alternative interpretations have been stressed that emphasize the widespread expression of many genes in a modular fashion in development of obviously unrelated organs (Gilbert and Bolker, 2001). While we are aware of this controversy, we feel it is important to provide a simplified snapshot of what is currently known of the incompletely understood molecular and structural history of the mechano-sensory organs across phyla. Naturally, molecular data in vertebrates far outweigh those in invertebrates generating the apparent impression that

most of these molecular combinations are new to vertebrates. We therefore also stress that absence of information should not be taken as indication of evolutionary discontinuity. Rather, plausible scenarios should be formulated that guide proper research to provide needed data to confirm or reject such ideas. We hope that more data will help clarify these complex issue but controversies may remain unresolved as is the case in molecular taxonomy (Rokas et al., 2005).

2. The cellular and molecular basis of mechanosensory cells

2.1. Structure, function and development of vertebrate hair cells

The specialized apex of hair cells of the inner ear and lateral line system of vertebrates is characterized by an array of actin filled microvilli (called stereocilia) which together form the hair bundle (Fig. 1, insert 1). The hair bundle is polarized, with the stereocilia arranged in rows that decrease in height away from the single kinocilium. The morphological polarity of the hair cell bundle enables a hair cell to transduce mechanical stimuli into electrical signals. To do this, stereocilia are interconnected with various links (Delprat et al., 2005; Michel et al., 2005; Sotomayor et al., 2005) that are thought to activate the mechanosensory channel which may belong to the transient receptor potential (TRP) channel family (Corey et al., 2004; Nicolson, 2005). Beyond the putative mechanotransducer channels, other molecules crucial for mechanotransduction in hair cells, are conserved across phyla even in morphological dissimilar structures. Such molecules are unconventional myosins (Todi et al., 2005), cadherins (Michel et al., 2005; Nicolson, 2005) and PDZ containing proteins (Delprat et al., 2005; Goodyear et al., 2005). Most of the numerous molecules necessary for vertebrate hair bundle formation are largely unknown in invertebrates such as flies (Boekhoff-Falk, 2005; Caldwell and Eberl, 2002; Fettiplace and Hackney, 2006; Muller and Littlewood-Evans, 2001) thereby generating a bias.

Development of mammalian hair cell bundles starts with a central kinocilium surrounded by short microvilli. As development progresses, the kinocilium moves into an asymmetric position and microvilli transform into stereocilia (Barald and Kelley, 2004; Dabdoub et al., 2003). A dynamic process regulates the differential length of stereocilia (Rzadzinska et al., 2005). Mammalian cochlear hair cells lose the kinocilium during development.

Development of the hair cell is initiated by the bHLH gene *Atoh1* (Bermingham et al., 1999; Fritzsche et al., 2005a) and other genes are necessary for completion of development of hair cells in both fly and mice (Ben-Arie et al., 2000; Fritzsche and Beisel, 2004; Hertzano et al., 2004). In addition, hair cells are secondary sensory cells that lack an axon and instead transmit their information through neurons derived from the ear to the CNS (Fritzsche et al., 2002).

Evolution of hair cells raises several questions: (i) What are structural similarities of mechanotransducer cells across phyla? (ii) Can the alterations in apical specializations be variations of a common theme? (iii) Is there a molecular basis to assume 'deep homology' of structural molecules of all mechanosensory cells? (iv) Is the development of mechanosensory cells molecularly conserved across phyla? (v) Are hair cells unique among secondary sensory cells in terms of the molecular basis of afferent innervation?

2.2. What are structural similarities of mechanotransducer cells across phyla?

The origin of the vertebrate hair cell has been discussed extensively and hair cells have been compared to invertebrate mechanosensory cells that may perform similar functions (Caldwell and Eberl, 2002; Fritzsche and Beisel, 2004; Jorgensen, 1989; Mackie and Singla, 2004). Apparently deep-rooted similarities have been used to argue in favor (Burighel et al., 2003) or against (Holland, 2005) detailed homology. Comparisons among all phyla show

that the collar cell, a receptor cell with a central cilium surrounded by microvilli, is present in most basic metazoa as either statocyst or epidermal (hydrodynamic) cells (Budelmann, 1987, 1989; Jorgensen, 1989). Close examination shows that the basic organization of a developing hair cell, a cilium surrounded by microvilli, is comparable to the putative single cell ancestor of all multicellular animals, the Choanoflagellates (Jorgensen, 1989; King, 2005; King and Carroll, 2001; Lavrov et al., 2005; Orme et al., 2001; Philippe et al., 2004). Indeed, recent molecular data strongly support that some protists together with animals and fungi form a single distinct taxon, the Ophisthokonta (Steenkamp et al., 2006). This outgroup comparison supports the notion that a cilium surrounded by microvilli was present in the metazoan ancestor (Fig. 1). Moreover, the actin filaments in microvilli/ stereocilia of invertebrates/vertebrates and tubulin of motile and non-motile cilia/kinocilia of invertebrates/vertebrates are both conserved among eukaryotes (Jensen-Smith et al., 2003; Nielsen and Raff, 2002). It is therefore possible that formation of a non-motile cilium surrounded by a symmetric or asymmetric bundle of microvilli as found in diploblasts (Arkett et al., 1988; Budelmann, 1989; Jorgensen, 1989) and all basic triploblasts is as old as diploblastic animals and therefore predates the evolution of deuterostomes and thus chordates (Fig. 1). Indeed, diploblasts have both hydrodynamic single surface receptors as well as statocysts studded with receptor cells that bear a single cilium surrounded by a collar of thick microvilli sometimes referred to as 'stereovilli' (Budelmann, 1989). Some of these 'stereovilli' have microfilament rootlets comparable to the actin rootlets of hair cell stereocilia. In addition, these microvilli may be asymmetric in some species (Arkett et al., 1988) and possess links that can be repaired by proteins also able to repair lateral line organ hair bundles (Repass and Watson, 2001). Unfortunately, whether the links are molecularly comparable between diploblasts and vertebrates is unknown and the morphological polarity of these receptor cells has not been verified physiologically.

We propose that Choanoflagellates may not only closely resemble the protist ancestor of multicellular animals (King and Carroll, 2001; Lavrov et al., 2005; Philippe et al., 2004) but may also be the *universal common ancestral sensor* (UnCAS) of mechanosensors, an idea reminiscent of a proposal for photoreceptor evolution (Fritzsche and Piatigorsky, 2005b; Gehring, 2005). A central cilium surrounded by microvilli is the ancestral feature of all mechanosensory cells of metazoans (Fig. 1). The development of a central kinocilium that later becomes asymmetric (Dabdoub et al., 2003; Jorgensen, 1989) may recapitulate during vertebrate hair cell maturation the mechanosensory cellular evolution. While simple Haeckelian recapitulation has been refuted for many details initially suggested, the developmental events leading to gastrulation and formation of anus and mouth are still used as major taxonomic divisions, in analogy to our idea of how ancestral features may be conserved during evolution of ciliated cell development. If this hypothesis about the ancestry of ciliated sensory cells were true, we should be able to derive all metazoan mechanosensory cells from such a hypothetical ancestral cell.

2.3. Can the alterations in apical specializations be variations of a common theme?

Undoubtedly, mechanosensory cells vary greatly among metazoa in their apical specializations (Holland, 2005). Among vertebrates, dramatic variations in cilia/kinocilia and microvilli/stereocilia are known for electroreceptive cells that develop from the same lateral line placodes that give rise to the hair cells of neuromasts (Northcutt et al., 1995). Electro-receptive cells are not mechanoreceptive and thus have no selection pressure on maintaining the polarized hair bundle. Indeed, electroreceptive cells have either microvilli or a kinocilium or a mixture of both as apical specializations (Bullock and Heiligenberg, 1986; Jorgensen, 1989), apparently randomly distributed among those vertebrates possessing this ancestral sense. These data show that superficial similarities and even similarities in development do not establish similarities in function. It also raises the major problem of lack

of direct evidence for the alleged function of many suspected invertebrate mechanosensors, in particular those in non-vertebrate deuterostomes (Fig. 1). In conclusion, it is unclear whether some of the variation in apical specialization of suspected mechanosensors indicates that cells are sensing some other stimuli as chemosensory cells are also characterized by cilia and microvilli (Laverack, 1988).

Among non-vertebrates, the most obvious deviations from the collar cell are found in the ecdysozoan and lophotrochozoan apical specializations as both lineages have reduced the microvilli (Fig. 1). In ecdysozoans, a single cilium becomes modified (Fig. 1) to serve as the mechanotransducer (Boekhoff-Falk, 2005; Todi et al., 2004) and cephalopod mechanosensory cells have links between the many cilia (Fig. 1) that are reminiscent of vertebrate stereocilia links (Budermann, 1989). Some metazoan lineages have directional sensitivity of mechanical stimuli (Boekhoff-Falk, 2005; Budermann and Williamson, 1994) through what appear to be uniquely derived morphological adaptations of lophotrochozoans, ecdysozoans and vertebrates (Fig. 1).

Overall, these data suggest that metazoan mechanosensors show variations of cilia/kinocilia and microvilli/stereocilia (Fig. 1). These variations have been interpreted as either variation of a common theme (Budermann, 1989; Burighel et al., 2003; Jorgensen, 1989; Mackie and Singla, 2004) or as uniquely derived features (Holland, 2005). We agree with the later interpretation, but believe that all mechanosensory cells are interconnected through 'deep molecular homology'. Below we provide some evidence for this assumption.

2.4. Is there a molecular basis to assume 'deep homology' of structural molecules of all mechanosensory cells?

Molecular data on mechanosensory transduction, including mechanosensory channels and associated structures, are available only for flies, *C. elegans* and vertebrates (Boekhoff-Falk, 2005; Caldwell and Eberl, 2002; Nicolson, 2005), but whole genome sequencing adds greatly to our understanding of molecular evolution (Eichinger et al., 2005). The available molecular data allow one to compare the most morphologically dissimilar receptors (fly and mammalian auditory receptors; Fig. 1), thus critically testing the idea of 'deep homology'. Despite the obvious structural differences of fly auditory organs and vertebrate cochlear hair cells, molecules such as specific rare myosins are essential for auditory function in man and fly (Boekhoff-Falk, 2005). Most importantly the mutation of Myo VII results in deafness in flies, mice and humans (Todi et al., 2005). Rare myosins are therefore functionally relevant, ancestral features of triploblast mechanosensors. Myosins predate even the origin of metazoa and rare myosins, including MyoVII, are found in single celled slime molds (Eichinger et al., 2005) and plants where they may participate in anchoring membrane-bound elements to the actin core. Thus, while unique effects of this protein are shared in flies, mice and man, the actual function of this molecule may predate the evolution of mechanosensory cells. The unique effects on hearing loss in MyoVII mutations in mice, man, and flies may relate to the exceptional level of expression in these mechanosensory cells rather than present a unique function of this protein in these cells.

In addition, to rare myosin and housekeeping genes such as actin and tubulin, the actin binding molecule espin has recently been shown to be involved in stereocilia maturation (Rzadzinska et al., 2005), a unique derived feature of vertebrate hair cells. Espin is also ancestral to triploblasts as an espin homolog (forked) is known in the fly. Moreover, the genome of the slime mold has cell adhesion molecules previously thought of as unique to metazoa or even vertebrates, including cadherins relevant for hearing in flies and humans (Todi et al., 2004). Together, these molecular data suggest that specific aspects of the molecular basis for mechanoreception were present in the common unicellular ancestor and were likely simply recruited to serve a specific function in hearing.

Beyond these similarities, both fly and vertebrate mechanosensors seem to require similarities in ion composition although the details are not available for insect auditory organs (Boekhoff-Falk, 2005). Fly and vertebrate receptors seem to require related TRP channels for mechanotransduction but how close the similarities are remains unclear in the absence of identification of the mammalian mechanotransducer channel (Nicolson, 2005). Moreover, TRP channels are also found in mechanosensory cells that are not related to hair cells thus greatly complicating the use of these receptors to readily establish 'deep homology'. Directional perception of mechanical stimuli has been resolved in morphologically distinct ways for various animals (Budelmann, 1992; Budelmann and Bleckmann, 1988; Burighel et al., 2003; Jorgensen, 1989; Mackie and Singla, 2004). Despite such morphological differences (Fig. 1), development of directionality of these apical specializations depends on molecular mechanisms to set up cell polarity that are ancestral to triploblasts (Dabdoub et al., 2003; Davies et al., 2005). One such family of molecules are the WNT signals (King, 2004). WNTs are found in diploblasts (Martindale et al., 2004) and an essential component of WNT signaling, β -catenin, is known for slime molds (Eichinger et al., 2005). Several other genes necessary for the development of cell polarity are also conserved across phyla (Barald and Kelley, 2004) and are common at least to triploblasts.

Much like many other genes formerly thought to be associated selectively with triploblasts, genes once thought to be unique to hair cells are now identified in diploblasts (Rieger et al., 2005) or even single cell organisms (Eichinger et al., 2005; King, 2004). Unfortunately, hardly any experimental data on their function prior to the association with mechanosensors are available. Nevertheless, the current set of data is most easily compatible with the idea of mechanosensory cells being related through 'deep molecular homology'. It is to be expected that genes formerly considered to highlight uniquely derived structures of a given phylum will be found to be more widespread in other lines and involved in unrelated functions. Sorting this problem of molecular and structural antiquity from the derived features apparently characterizing a given lineage requires establishing molecular developmental networks tied into unique morphological features of a given lineage. Beyond gene description, we need to establish unique functional relationships associated with specific morphologies to fully resolve whether mechanosensory cells share 'deep homology' and are more than superficially similar.

2.5. Is the development of mechanosensory cells molecularly conserved across phyla?

At a molecular level, generating single cells or organs specialized in, for example, mechanical stimulus reception, requires specializing epithelial cells to acquire a sensory cell phenotype. Such novel cell fate acquisition was likely achieved by the expression of a basic Helix–Loop–Helix (bHLH) gene. bHLH genes are associated with cellular differentiation in plants, fungi and metazoans (Chapman-Smith et al., 2004; Ramsay and Glover, 2005; Tuncher et al., 2004). A diploblast example is the coelenterate *Achaete–Scute*-like bHLH gene that is involved in epidermal sensory cell development (Hayakawa et al., 2004). Such conservation of genes is particularly obvious in the *atonal* family of bHLH genes (Fritzsche and Beisel, 2004; Seipel et al., 2004) that can be exchanged between different phyla with a remarkable conservation of function (Wang et al., 2002). Indeed, past research of the development of mechanosensory fate specification has demonstrated that a small set of highly conserved transcription factors determines the developmental transformation of one or more ectodermal cells into mechanosensory cells (Caldwell and Eberl, 2002; Dong et al., 2003; Fritzsche and Beisel, 2004; Hertzano et al., 2004; Wallis et al., 2003; Wang et al., 2002). Specifically, these data imply that a conserved bHLH gene, *atonal* (fly) or *Atoh1* (previously *Math1*, vertebrates) specifies development of the mechanosensory cells present in insect auditory systems and vertebrate hair cells (Fig. 2). As previously pointed out

(Fritzscht and Piatigorsky, 2005b), a second mammalian orthologue of *atonal* (*Atoh7*, previously *Math5*) is found in retinal ganglion cells and suggests that *atonal* function in flies has been split into a mechanosensory and visual function.

More recent data have also shown that individual sensory cells in coelenterates are also specified by an *atonal* orthologue that shows sequence similarities to *atonal* and *Atoh1* (Seipel et al., 2004). This indicates that bHLH genes are both developmentally and evolutionary linked to the generation of diverse cell types in multicellular animals and likely existed in the metazoan ancestor (Figs. 2, 3). Moreover, three other genes are essential for hair cell maintenance: the Pou domain factor *Pou4f3* (Xiang et al., 2003), the zinc finger gene *Gfi1* (Wallis et al., 2003) and the homeodomain genes *Barhl1* (Li et al., 2002). In addition, these genes are also found in insect sensory development and *Barhl1* is immediately downstream of *Atoh1*. Combined with the increasingly data on other transcription factors, it appears that metazoan ancestor may have had already much of the transcriptional machinery of triploblasts (Rieger et al., 2005). Further evolution relied mostly on gene duplication in combination with sequence and regulation modification to generate the multitude of cells in the nervous system of vertebrates (Fig. 3).

Single bHLH genes, like *atonal* and *Atoh1*, are mutually exchangeable. Complete restoration of normal mechanosensory cell development in either fly or mouse is driven by the orthologue gene (Grens et al., 1995; Wang et al., 2002). Such developmental compatibility requires that the downstream activation cascade of *atonal* and *Atoh1* is conserved to the extent that a fly gene can replace a mammalian gene and vice versa. It is now clear that the jelly fish orthologue, *Atonal-like 1* (Seipel et al., 2004), plays a role in both muscle and sensory fiber selection. Muscle fiber differentiation in vertebrates critically depends on the MyoD bHLH gene family as these members have the capacity to bind to *E-boxes* of the muscle myosin promoters (Fig. 3). It is notable that vertebrate hair cells and insect mechanosensory cells are characterized by several rare myosins (Fritzscht and Beisel, 2004). Screening the promoter regions of the rare myosins expressed in the mouse hair cells has not shown obvious *E-box* binding sites (unpublished data). However, it is entirely possible that other immediate downstream genes of *atonal/Atoh1* such as *Barhl/BarH* or genes co-expressed with *atonal/Atoh1* or the zinc finger protein *senseless/Gfi1* or *Pou4f3* (Clough et al., 2004; Hertzano et al., 2004; Li et al., 2002; Wallis et al., 2003; Xiang et al., 2003) are required to fully develop a mechanosensory cell. Rare myosins are not expressed in the undifferentiated hair cell precursors that form in *Atoh1* null mouse ears (Bermingham et al., 1999; Fritzscht et al., 2005a; Xu et al., 2004). Interestingly, slime molds have numerous rare myosins but lack bHLH genes entirely (Eichinger et al., 2005). It is therefore possible that regulation of rare myosins in hair cells does not depend on bHLH genes and the apparent absence of expression in *Atoh1* null mice (Bermingham et al., 1999) may reflect simply the undifferentiated nature of the remaining cell expressing an *Atoh1*- β -galactosidase marker (Fritzscht et al., 2005a).

We conclude that the ‘deep homology’ for insect mechanosensors and vertebrate hair cells is strongly supported by numerous co-expressed transcription factors. Unfortunately, the possibly conserved function of *atonal/Atoh1*, namely to regulate some of the genes necessary for mechanosensory transduction, is not known at present. Overall, the most simplistic interpretation of the current set of structural and molecular data is that mechanosensory cells are ancestral cells of metazoa, but that crown groups (the living relatives of ancestral taxa) each show unique derived variations of the ancestral theme (Fig. 1).

2.6. Are hair cells unique among secondary sensory cells in terms of the molecular basis of afferent innervation?

Sensory cells in vertebrates either have an axon (primary sensory cells such as olfactory receptors) or are innervated by placode derived neurons associated with “secondary” sensory cells such as inner ear, lateral line, electroreceptor, taste bud sensory cells; Fig. 2. In the ear and the lateral line, neurons innervating these sensory cells develop from the same placodes that give rise to hair cells, and some evidence for a clonal relationship for both cell types exists for chicken and mammalian ears (Matei et al., 2005; Satoh and Fekete, 2005). Development of inner ear neurons depends critically on the bHLH genes *Neurog1* (Ma et al., 2000; Matei et al., 2005) and *Neurod1* (Kim et al., 2001; Liu et al., 2000). Expression of *Neurog1* in the ear is conserved among vertebrates but not in other placodally or neural crest derived neurons (Furlong and Graham, 2005). However, *Neurog1* and *Neurod1* are also necessary for olfactory receptor development in mice (Kawauchi et al., 2004) thus indicating that *Neurog1*, while necessary for ear placode derived neuron formation, is not sufficient to identify such neurons. Placodally derived sensory neurons use the Pou domain factor *Pou4f1* (*Brn3a*) to upregulate the neurotrophin receptor *Ntrk2* for survival via the neurotrophin *Bdnf* released from their target, the hair cells (Fritzsche et al., 2004). Among peripheral neurons, ear neurons of mice can be uniquely identified by the sequential expression of *Neurog1*, *NeuroD1*, *Pou4f1* and *Ntrk2* in combination with other factors such as *Gata3* (Karis et al., 2001). Whether this is true for other vertebrates is unclear.

Most invertebrates have primary receptors with their own axon. However, in several taxa some cells exist that have no axon (Jorgensen, 1989; Mackie and Singla, 2004). Secondary sensory cells have been described in amphioxus, but neither their innervation nor their function is known (Holland, 2005). Secondary sensory cells have recently been described in some ascidians (Burighel et al., 2003) but the location of the neurons that innervate these mechanosensory cells is unknown. Structurally most similar to vertebrates are the secondary sensory cells in cephalopod mollusks that are innervated by adjacent neurons that project to the central nervous system (Budelmann, 1992). Neither the origin nor the molecular basis of development of these neurons is known. It is therefore possible that all of these similarities represent cases of parallel evolution from an ancestral primary mechanoreceptor cell.

Molecular data are not helpful to resolve these issues. Neurogenin genes are found in flies (*tap* or *biparous*), ascidians (Satou et al., 2003) and amphioxus (Holland, 2005) and are thus ancestral to triploblasts. *Neurogenin* genes have not been described in coelenterates (Martindale et al., 2004; Seipel et al., 2004) but there is no complete genome sequence of coelenterates available as yet. *Neurogenin* is expressed in amphioxus in several sensory cells anterior to the mouth and in the central nervous system (Holland, 2005) but whether these cells are primary or secondary sensory cells is unknown. Interestingly, sensory cells that express neurotrophin receptors are also present in amphioxus (Benito-Gutierrez et al., 2005). The ligand is unknown and it is unclear why the receptor should be expressed in sensory neurons in the skin where ligands are expressed in vertebrates (Huang and Reichardt, 2001b). A *neurogenin* gene has been found in ascidians (Furlong and Graham, 2005) but its expression is unknown and no neurotrophin receptors have been identified in the genome of *Ciona*. In insects, *neurogenin* is found in sensory neurons and glia cells but neurotrophins or neurotrophin receptors have not been identified.

In summary, neither the distribution of neurons innervating secondary sensory cells nor the distribution of molecules essential for inner ear sensory neuron development is known well enough to suggest the existence of such neurons outside craniate vertebrates. The available data suggest that *Neurog1* association with neurons innervating hair cells is a derived feature of craniate vertebrates. The upregulation of *Neurog1* early in ear development is a radical alteration of the proposed evolutionary history: a gene unique to triploblasts (*Neurog1*) is

expressed at an earlier stage in development than the genes of a developmental program that generates mechanosensory cells in triploblasts (*atonal/Atoh1*). Moreover, *Neurog1* is not only heterochronically expressed with respect to *Atoh1* in mice but it directly affects development of hair cells (Matei et al., 2005).

The most parsimonious interpretation of the current data is that mechanosensory cells across metazoa are derived from an ancestral diploblast or pre-metazoan cell. The unique features found in various lineages are variations of a common theme that is more likely to reflect 'deep homology' rather than 'homology' in a strict morphological sense. Vertebrate hair cells are uniquely derived. The molecular similarities with the structurally dissimilar fly receptor cells may reflect the 'deep homology' among metazoan mechanosensors. Neurons that innervate mechanosensors are unique for vertebrates and can not be compared with neurons innervating secondary sensory cells in invertebrates, unless placodal origin and the necessity of neurogenin for their development are shown. Vertebrate hair cells are in addition innervated by efferent fibers derived from facial branchial motoneurons (Karis et al., 2001), another component of likely parallelism to other metazoa having such efferent innervation (Budelmann, 1989; Burighel et al., 2003). The inner ear hair cell and its innervating neuron is therefore an apomorphic feature unique to vertebrates that shares only plesiomorphic structural and molecular features with invertebrate mechanosensors (Figs. 1–3).

3. Evolving multicellular mechanosensory organs

3.1. General considerations of multicellular organ formation

All metazoans have both single sensory cells as well as arrays of sensory cells. Parsimony argues that single sensory cells are ancestral to arrays of sensors. The alternative that arrays of sensors in complex organs is ancestral and single cells are derived is rejected here as an overly complex scenario. Making arrays of sensory cells instead of single cells and turn them into organs requires (i) multiplication of each sensory/support cell unit and (ii) aggregation of these cells to generate organized groups of receptors in specific localities. Understanding multiplication of sensory cells requires understanding how the molecular process to select a single cell out of the epidermis to differentiate into a sensory cell is multiplied to form sensory cell arrays. As a byproduct of such aggregation, the diffuse distribution of single sensory cells to coalesce into an organ has to be tied into the global patterning processes of the embryo to specify the more restricted location of sensory patches or organs.

Central to the problem of forming increasingly larger and more complex organs, such as mechanosensory organs found in several multicellular animal lines (Budelmann, 1989; Markl, 1974), is the need to expand the epidermal anlage rapidly. This can be achieved by using cell cycle regulatory mechanisms that allow symmetric division for rapid increase of undifferentiated aggregates of cells, referred to as placodes in several phyla (Fritzscht and Beisel, 2004; Schlosser, 2005). Such accumulations of highly proliferative but as yet unspecified precursors typically form a placode that undergoes invagination thus generating an internalized organ such as an otocyst. Forming such large organs necessitates the evolution of a molecular system that allows retention of proliferation without differentiation; such placodal cells therefore should bear characteristics of stem cells. A vertebrate module for stem cells has recently been defined and consists in its minimal form of three interactive transcription factors, OCT4 (POU5F1), SOX2 and NANOG (Boyer et al., 2005). Unfortunately, *Oct4* null mice are early lethal as they can not implant into the uterine endometrium. In contrast, *Sox2* mutants are viable and present a severe ear phenotype, the yellow submarine (Kiernan et al., 2005). Likewise, the neuronal phenotype of *Sox2* has not been described in the ear, but a severe reduction, if not loss of sensory neurons, can be

expected. *Nanog* has not been studied in the ear nor has an ear phenotype of mutants been described. Recent data on zebrafish have shown that *Neurog1* expression critically depends on Pou2/Oct4 and disappears in Oct4 null zebrafish (Reim and Brand, 2002). This suggests that one of the earliest genes to be upregulated in the ectodermal transition to become otic ectoderm is the proneural gene that is essential for sensory neuron formation.

Extensive molecular data on transcription factors related to this core transcriptional module for stem cells have been identified (Boyer et al., 2005) and some of those immediate downstream genes have been observed in placodes (Holland, 2005; Schlosser, 2005). One of the downstream genes common to *Oct4*, *Nanog* and *Sox2* is *Neurog 1* (Boyer et al., 2005). In the ear, *Neurog1* is expressed very early and is essential for sensory neuron and some hair cell development (Ma et al., 1998, 2000; Matei et al., 2005). In addition to the upregulation of *Neurog1*, there appears to be the need to upregulate the bHLH genes *Hes1*, *Hes3* and *Hes5* to fully realize the proliferative capacity of progenitors (Kageyama et al., 2005). *Hes1* and *Hes5* are known to be relevant for ear histogenesis (Zine et al., 2001).

Members of these gene families are found in triploblasts, suggesting that the molecular basis for placode formation as an embryonic adaptation for rapid proliferation is an example of 'deep homology' and that any attempt for a more detailed 'homology' among placodes is likely to fail. This assertion is underscored by the simple fact that placodes are widespread among vertebrates and invertebrates. Placodes can be grouped into three categories: non-neuronal placodes (lens placode of vertebrates, trachea placode of insects), neuronal placodes that give rise to neurons (epibranchial placodes), neuronal placodes that give rise to sensory neurons (olfactory placode) and neuronal placodes that give rise to neurons and sensory cells (otic and lateral line placodes). Among placodes, the otic and lateral line placodes are uniquely derived and present a vertebrate feature not shared with invertebrates (Holland, 2005; Schlosser, 2005). All cranial ganglia may be derived from placodes in lampreys, but not largely from neural crest as in gnathostomes (McCauley and Bronner-Fraser, 2003). Indeed, placodes are embryonic adaptations that reflect the need to maximize proliferation in a restricted epithelial area that is subsequently specified to become the ear or other placodally derived structures. While attempts have been made to exclude such ectodermal thickenings of invertebrates (Schlosser, 2005), such attempts may be in need for revision as more detailed information on molecular similarities across these embryonic adaptations of vertebrates and invertebrates are found. Most importantly, there is hardly any experimental evidence for the function of most genes in animals other than mice and zebrafish, thus rendering ideas about conserved function of such genes across phyla highly tentative.

To complicate matters, some of these transcription factors are pleiotropically used for development of other organs and are also involved in global or local patterning processes. Thus it remains unclear whether, for example, distal-less homeobox (*Dlx*) expression in the ear is primarily related to the conservation of the core transcription system to maintain stem cells or has been independently recruited. The known major function of these factors in ear development notwithstanding, it seems to be important not to use those genes to assess 'homology' across phyla as they could be expressed as a component of generalized developmental proliferation module or where independently co-opted (Gilbert and Bolker, 2001; Meulemans et al., 2003). This is a problem for several genes known to be important in ear development (Adamska et al., 2001; Burton et al., 2004; Huang et al., 2001a; Merlo et al., 2002; Riccomagno et al., 2002; Xiang et al., 2003; Zou et al., 2004).

Only few developmental genes are both expressed in otic placodes and have also been experimentally proven to be essential for placodal differentiation across vertebrates. The closest candidates are *Fgf3/Fgf10/Fgf8* and their receptor *Fgfr2b/Fgfr3* are all essential for

ear formation in mice, chicken and zebrafish (Ladher et al., 2005; Pauley et al., 2003; Pirvola et al., 2000; Wright and Mansour, 2003a) but are not exclusive to the ear placode. Other candidates are the Fox genes, in particular *Foxi* genes. However, their essential function across vertebrate ear formation appears not to be conserved (Hatini et al., 1999; Hulander et al., 2003; Solomon et al., 2003). Unfortunately, these genes have not been analyzed in great detail in invertebrates and given their molecular antiquity (Martindale et al., 2004) they may prove to be another example of ‘deep homology’.

Further diversification of placodes could be generated from ancestral placodal templates by using various members of a given transcription factor family combined with newly recruited genes to modify the outcome. Overlapping expression of such unique combination of several transcription factors may provide the transcriptional novelties required for evolution of an apparently new organ such as the vertebrate ear. Some of these apparently homologous genes in flies and mammals (Table 1) are possible examples of ‘deep homology’ as the resulting morphology is highly dissimilar in those two phyla.

3.2. Statocyst and hydrodynamic receptors of invertebrates

The evolutionary origin of the vertebrate ear as a three dimensional structure is unknown, but various statocysts and hydrodynamic organs composed of mechanosensory cells exist among invertebrates (Budelmann, 1989; Markl, 1974). Major distinctions between those two receptor types are the presence of denser material for linear acceleration perception in statocysts or a gelatinous cupula for enhanced hydrodynamic reception (i.e., water movement across the surface). Outgroup comparison shows that the molecular basis for extracellular material formation including calcification is found in single celled organisms (Fig. 3) and is therefore ancestral to metazoa (Eichinger et al., 2005; King, 2004). The structural differences between invertebrate gravistatic and hydrodynamic receptors suggest that these organs are uniquely derived in each lineage and that molecular similarities reflect convergent use of ancestral molecules, that is, these organs are likely a case of ‘deep homology’ at the cellular level but of convergence at the organ level.

Outgroup comparison shows that multicellular statocysts, which function as gravity receptors using otoconia like structures, are widespread among free swimming marine animals. Multicellular hydrodynamic receptors are less widespread. Whether mechanosensory cells of statocysts functioned at the single cell level as gravistatic cells or are derived from hydrodynamic sensors remains unclear. Other than vertebrates only the mollusk cephalopods have evolved a statocyst that combine a hydrodynamic function for angular acceleration perception with gravistatic function (Budelmann, 1992). However, there is no known evolutionary sequence showing hydrodynamic receptors to be converted to gravistatic receptors. Such a conversion is unlikely since hydrodynamic receptors would be rendered functionless upon invagination into a closed sack. The original vertebrate ‘ear’ (Markl, 1974) therefore likely was a statocyst, not a hydrodynamic organ. We suggest that vertebrate ears evolved in two steps: first the formation of a statocyst-like gravistatic organ covered by otoconia, followed by the formation of canal cristae that lack otoconia. As previously pointed out, only fast moving aquatic animals evolved semicircular canal like structures (Budelmann, 1987). We consider an ear consisting of a single gravistatic sensor and two angular sensors to be the minimal essential array needed for angular and linear acceleration perception.

Various proposals have been made as to which chordate species may have an ancestral organ homologous to the vertebrate ear. (Burighel et al., 2003; Lacalli, 2004; Schlosser, 2005). However, it has to be stressed that both amphioxus and free swimming ascidians have gravity receptors inside the brain at least as larvae (Lacalli, 2004) and thus do not have a strong selective pressure to form a bilateral gravistatic receptor outside the brain. In other

words, the formation of a gravistatic receptor outside the brain (e.g., the vertebrate ear) evolved in parallel to the intracerebral gravity receptors of other chordates. As pointed out by others (Coffin et al., 2004; Holland, 2005), certain structural and topological similarities, even if combined with expression patterns of some ear relevant genes, do not specify an ear as we know it in vertebrates for an invertebrate. Organs with primary or secondary sensory cells outside the brain of ascidians (Burighel et al., 2003) apparently function as hydrodynamic and not as gravistatic receptors (Budelmann, 1989) thus rendering their transformation into a statocyst unlikely. Whether the many distributed single and multiple sensory cells in the skin of amphioxus (Holland, 2005) and the acorn worm (Lowe et al., 2003) are at all related to the lateral line or inner ear of vertebrates remains an open question.

3.3. Evolution of the vertebrate ear shows three main changes

We assume here that the evolution of secondary mechanosensory cells to form hair cell arrays predates the evolution of the vertebrate ear (Fig. 4). The vertebrate ear, however, is considered as a novelty that arose in conjunction with the craniate brain formation and other major senses. The unknown cellular precursors may have had only some topographical resemblance among invertebrates in terms of homologous transcription factor expression that specify the topology (Lowe et al., 2003) and regulate proliferation (Boyer et al., 2005). Indeed, much like the vertebrate brain can be viewed as the aggregation and centralization of the general neuronal capacity of the ectoderm (Holland, 2003), formation of the vertebrate ear may also require the condensation of the general capacity of the skin to form sensory neurons only into a single area, the cranial placodes.

A possible morphological prototype of the vertebrate ear is represented by the ear of the hagfish (Fig. 4). These animals have a single torus with three sensory patches (Jorgensen et al., 1998). Two sensory patches, known as anterior and posterior crista, have mechanosensory hair cells that surround the canal lumen and project long kinocilia and stereocilia into the endolymph-filled lumen. These organs do not have a cupula covering the canal epithelia. The third epithelium of this ear has otoconia that are, however, structurally distinct from those of other vertebrates (Lewis et al., 1985). The organization of the canals in a single torus likely represents an ancestral condition in hagfish. This suggestion is based on the absence of a cupula covering the canal crista and the organization of hair cells in a ring surrounding the lumen rather than as a single patch (Fig. 4). The organization of sensory cells in the canals of the hagfish is unlike any other hair cells found in vertebrates.

The uniqueness of the hagfish ear is further underscored by the presence of two octaval nerves, an anterior nerve supplying the anterior crista and the anterior two thirds of the common macula and a posterior nerve supplying the posterior crista and the posterior one third of the common macula (Amemiya et al., 1985; Fritzscht, 1998; Retzius, 1884). Hagfish, lampreys and all gnathostomes have an efferent innervation of the ear that apparently has evolved with the formation of the ear (Fritzscht et al., 1989; Jorgensen et al., 1998). It appears less likely that the absence of cupulae and the presence of a posterior octaval ganglion are secondarily derived through loss of cupulae and addition of a ganglion in hagfish. In conclusion, the simple organization of the hagfish ear is consistent with the idea that it resembles the “prototype” of the vertebrate ear.

A more complicated ear with a total of four sensory epithelia is found in the sister taxon of hagfish, the lampreys. Indeed, recent molecular evidence strongly supports the once dismissed taxonomic entity of cyclostomes, i.e., hagfish and lampreys (Takezaki et al., 2003). In contrast to hagfish, lampreys have cupulae and the canal cristae are organized into multilobed patches (Hagelin, 1974; Lewis et al., 1985). The common macula bearing the otoconia is also partially split into discrete patches with different hair cell polarities

(Hagelin, 1974). An additional feature of lampreys is that the two canals form an obtuse angle and thus allow the detection of angular acceleration in two planes at a 90 angle in each ear. A fourth epithelium, the dorsal macula has no known function and resides next to two chambers with actively beating cilia that are unique for vertebrate ears (Popper and Hoxter, 1987). We propose that the dorsal sensory patch might have been coopted into the horizontal canal crista found in jawed vertebrates. The absence of the horizontal canal in modern cyclostomes is consistent with this interpretation and with fossil evidence which also suggests absence of a horizontal canal in jawless vertebrates (Mazan et al., 2000).

In contrast to cyclostomes, all jawed vertebrates have a horizontal canal. Evolution of a horizontal canal is embedded into a whole body reorganization that generated jaws, fins or limbs, alterations of eye muscle patterns, sympathetic and parasympathetic ganglia, and alterations of hindbrain Hox gene expression patterns (Fritzscht, 1998; Fritzscht and Northcutt, 1993; Fritzscht et al., 2001; Mazan et al., 2000; Murakami et al., 2004, 2005). The causality of all of these changes remains one of the unresolved puzzles in vertebrate evolution.

The craniate vertebrate horizontal canal system is structurally different from vertical canals. This canal system has its own sensory patch, a separate set of sensory neurons, a canal oriented at 90° angle with respect to the other two canals (Fig. 4), and a hair cell polarity different from the other canals (Fritzscht et al., 2002; Lewis et al., 1985). Evolution of a horizontal canal allows perception of horizontal head movements such as generated with lateral head movement during swimming. Combined with lateral fins, it allows a more stable swimming pattern than that generated by cyclostome vertebrates that lack this canal. The radical developmental and morphological reorganization required for the formation of jaws and limbs also resulted in the reorganization of eye muscles. Thus, these vertebrates evolved eye muscles with a different geometry of origin and insertion points and more significantly the formation of at least one new eye muscle to allow movement in the horizontal plane to be driven by the newly evolved horizontal canal input (Fritzscht, 1998).

While there is variation of the basic ear morphology found in jawed vertebrates, it is only the sarcopterygian line leading to land vertebrates that shows another radical change in the ear. Possibly starting with the coelacanth, *Latimeria*, this line developed a novel epithelium that differed by its association with a perilymphatic duct (Fig. 4). Such a duct permits sound pressure to reach the sensory epithelia via induced perilymph motion, thus generating a water-based sound pressure detector (Fritzscht, 1992, 2003). Further evolution of this new mechanosensory system for sound pressure reception into the mammalian cochlea is reasonably well documented but not undisputed. Likewise, evolution of amphibian sound pressure detection epithelia has generated a wide array of opinions among researchers about homology which, however, seem to have settled as of late (Fritzscht, 1992; Fritzscht et al., 1988; Smotherman and Narins, 2004). The consensus appears to be that the amphibian papilla is derived from the neglected papilla and that the amphibian basilar papilla, despite minor structural differences, is homologous to the basilar papilla of other sarcopterygians and the cochlea of mammals. We and others have pointed out that the pattern of innervation is the most reliable evidence to uncover homology in a system with dramatic changes in shape and relative topology (Fritzscht et al., 1988; Retzius, 1884). Molecular analysis of pathfinding has shown that the conservation of inner ear innervation is deeply rooted in the genetic basis of pathfinding (Fritzscht et al., 2005b). We therefore like to reiterate that position and argue that the basilar papilla of *Latimeria*, amphibians, and amniotes is, based on its structure, topology and innervation, homologous to the cochlea of mammals. Much like molecular evolution builds on multiplication and diversification of existing genes (Fig. 3) so does the ear multiply and diversify endorgans (Fig. 4A). In contrast to molecular diversification, ear development apparently recapitulates the progressive segregation of a

single sensory patch (Fig. 4B) as does the developing hair cell recapitulate the ancestral central kinocilium position (Fritzscht et al., 2002; Jorgensen, 1989).

If the outlined scenario of vertebrate ear evolution is accepted, it leads to two major questions about ear morphogenesis:

1. What is the molecular developmental basis for the canal formation in general and the horizontal canal of jawed vertebrates in particular?
2. What is the molecular developmental basis for the formation of the vertebrate cochlea?

We recently provided new insights into the evolution of the auditory system, including the cochlea (Fritzscht et al., 2006) and therefore have focused in the present review on the first question.

4. The molecular basis for the canal formation in general and the horizontal canal of jawed vertebrates in particular

Morphogenesis transforms the otocyst into the three dimensional shape of the vertebrate inner ear, a labyrinth of ducts and recesses. Morphogenesis of the ear is accomplished, in analogy to other systems, in three interdependent steps: patterning the anlage, growing the small otocyst, and sculpting the form. Many mutations have been identified and their morphogenetic defects have been described (Chang et al., 2004b; Fritzscht, 1998; Kiernan et al., 2002; Romand et al., 2003). While still patchy, we can now deduce a flow of events and their interdependence for proper ear morphogenesis. We propose grouping ear morphogenesis genes into several major categories assigned to specific functions. We realize that genes may be sequentially expressed, with major and minor expressions changing over time. While keeping these caveats in mind, the following three broad categories can be established:

1. Genes that are expressed in the ear and exert a general function on overall capacity of the ear to develop. Candidates for such genes are *Eya1*, *Six1*, *Sox2*, *Gata3*, *Fgfr2b* and *Fgf3/10* or *Fgf3/8* (Karis et al., 2001; Kiernan et al., 2005; Ladher et al., 2005; Pauley et al., 2003; Pirvola et al., 2000; Zou et al., 2004). In essence, mutations in these genes lead to complete arrest at the level of the otic placode or otocyst. In addition, these genes may play specific functions in promoting proliferation or directing growth and their regulation may be at the root of effects caused by mutations in other genes more dedicated to patterning the ear.
2. Genes that are expressed primarily outside the ear and exert their effects either by affecting gene upregulation in the ear as diffusible substances within or outside of the ear. Diffusible factor genes or modifiers are *Wnt1/3a*, *Shh*, and *Gli3* (Deol, 1983; Riccomagno et al., 2002, 2005). *Kreisler*, *Gbx2*, *Hoxa* and *Hoxb* cluster genes are known to regulate the production of such diffusible factors in the brain (Bober et al., 2003; Deol, 1983; Lin et al., 2005). Other genes expressed in the inner ear, such as *Hmx2*, *Dlx6*, and *Pax2* can influence these factors (Bober et al., 2003; Chang et al., 2004b; Robledo et al., 2002; Wang et al., 2004). We recognize that some of these genes may be more complex in their action but for the sake of clarity it appears paramount to combine them into a logical flow diagram of effects (Fig. 5) and possible interactions (Fig. 6).
3. Genes that fine tune morphogenesis of the ear by regulating fusion plate formation of the canals and regulating size of the canal diameter. Examples of these genes are

Bmps, *EphB2*, *Nor1* and *Netrin* (Cowan et al., 2000; Ponnio et al., 2002; Salminen et al., 2000).

In the following sections, we want to highlight some of the cooperative pathways that have been worked out. We want to present first the dorsoventral patterning followed by the anterior–posterior patterning of the hindbrain. Essentially, we suggest that the patterning events of the hindbrain directly influence the otocyst as suggested in numerous older and some more recent transplantation and extirpation experiments (Bok et al., 2005; Fritzsche, 1998). It is interesting to note that most genes involved in dorsoventral patterning of the ear and hindbrain are involved in anterior–posterior boundary formation of *Drosophila* segments (Carroll et al., 2005) suggesting that an ancestral genetic module was used in these two phyla for patterning of different axes in flies and vertebrates. For easier understanding of this argument we have aligned fly and vertebrate genes (Table 1).

4.1. Dorso-ventral and anterior–posterior patterning of the ear and the brain

Sonic hedgehog (*Shh*), an orthologue of the fly gene hedgehog (*hh*), determines ventral fate in the brain (Litington et al., 2002). Hedgehog (*hh*) is expressed in engrailed positive cells that are adjacent to wingless expressing cells near anteroposterior segmental boundaries. The positive feedback regulation of *hh* by wingless and of wingless by *hh* generates a sharp cellular boundary (Carroll et al., 2005). In contrast to this short range interaction in flies, *Shh* and *Wnts* are expressed in the floor plate and roof plate, respectively, of the developing brainstem and spinal cord (Gowan et al., 2001; Litington et al., 2002; Muroyama et al., 2002; Ornitz and Marie, 2002; Xiang et al., 2003). *Wnt* genes are co-expressed with *Bmp4* and *Gli3*. Combined, *Bmp4*, *Gli3* and *Wnt* genes antagonize the ventralizing effects of SHH and bestow dorsal identity. This counter-gradient set up by these genes allows a detailed allocation of various downstream genes for regional identity (Fig. 6).

Anterior–posterior regionalization depends on the *Hox* cluster of genes (Escriva et al., 2002; Murakami et al., 2004). Others are the Kreisler genes known to affect also the expression of *Hox* and *Fgf3* genes (Wright and Mansour, 2003b). Given the proximity of the ear to the hindbrain, the patterning genes in the hindbrain will affect the ear development as shown in many classic experiments (Fritzsche et al., 1998). Likely, correlates for the evolutionary changes in ear morphology of vertebrates are variations in expression of *Hox* genes and the altered positions of the otocyst in lampreys and gnathostomes (Fritzsche, 1998; Murakami et al., 2004). As previously suggested, the evolution of the ear hinges on the relative position of the ear along the anterior–posterior axis, the proximity to the hindbrain, and the expression of dorsoventral as well as anterior–posterior genes (Fritzsche et al., 1998).

4.2. All patterning genes may serve to regulate the expression of genes such as FGFs and FGFRs that mediate their effects

The effects of the hindbrain via *Wnt1/3a* and *Shh* on the developing ear are comparable to the effect of these genes on the developing somites (Brent and Tabin, 2004; Brent et al., 2005; Riccomagno et al., 2002). Consistent with this similarity is the upregulation of FGFs and their receptors in somites under the influence of bHLH genes (Brent and Tabin, 2004). FGFs are mitogenic but also play roles in cellular differentiation, motility and survival. FGFs are involved in neuralizing the ectoderm (Colvin et al., 1996; Pauley et al., 2003; Pirvola et al., 2000), which likely includes neuralizing of the otic placode.

FGF factors play a pivotal role for mammalian ear formation and histogenesis as demonstrated by null mutants of *Fgf* genes and their receptors (Alvarez et al., 2003; Wright and Mansour, 2003b). Double null mice (*Fgf3*^{−/−} *Fgf10*^{−/−}) show only limited formation of occasional micro vesicles (Wright and Mansour, 2003a). Similarly, *Fgf3*/*Fgf8* knockdown (morpholino) zebrafish do not develop otic vesicles (Maroon et al., 2002) and both *Fgf3* and

Fgf8 are essential for placode formation in mice and chicken (Ladher et al., 2005). An ear forms in *Fgf3* mutant mice, but the size reduction of the ear is apparently different in two *Fgf3* null lines, ranging from vesicle-like (Mansour, 1994) to apparently normal (Alvarez et al., 2003). Smaller ears also form in *Fgf10* null mice (Ohuchi et al., 2000) and *Fgf10* null ears show defective morphogenesis (Ohuchi et al., 2005; Pauley et al., 2003). FGF3 and FGF10 proteins interact in a dose dependent fashion to reduce ear vesicle size (Wright and Mansour, 2003a). In *Fgfr2b* null mice, the receptor for both FGF3 and FGF10, ear morphogenesis is almost entirely lost (Pirvola et al., 2000). The effect of *Fgf10* is less severe on ear morphogenesis due to signaling redundancy by other FGFs. *Fgf10* null mice do not develop semicircular canals, which is consistent with FGF's role in branching morphogenesis (Pauley et al., 2003).

There is an initial generalized expression of some FGFs throughout the ear, which is succeeded by specific expression in some sensory epithelia and sensory neurons (Alsina et al., 2004; Ohuchi et al., 2005; Pauley et al., 2003; Pirvola et al., 2000; 2002; 2004; Wright and Mansour, 2003b). *Fgf3*, *Fgf10* and *Fgf8* are expressed in areas of known early upregulation of bHLH genes, such as *Neurog1*, *Neurod1* and *Atoh1* (Kim et al., 2001; Ma et al., 1998, 2000; Matei et al., 2005). Specific FGFs mediate their signals through specific FGF receptors. Therefore, the ear needs to regulate the expression of FGFs and FGFRs. In addition, the molecular basis for FGFR regulation has to be different from that for FGF regulation otherwise a simple autocrine loop would be generated. In analogy to the developing fly trachea, several vertebrate homologues are needed to regulate *Fgfr* expression in the ear (Table 1).

It is conceivable that the growth of the canal plate is directed by the distribution of FGFRs and diffusion rates of FGFs in combination with BMPs (Chang et al., 2004a). Following the directed vertical canal growth, sculpting needs to eliminate the cells between the plates to form the definitive canal along with their variable radius and shape. This process needs to be flexible to support the various proportions of radius to diameter recognized among different vertebrates (Lewis et al., 1985). Four molecular components of this process are known. *Netrin* is apparently directly involved in directed plate growth and morphogenesis of the overall canal radius (Salminen et al., 2000). *EphB2* and *Nor1* are responsible for the final diameter of the canals (Cowan et al., 2000; Ponnio et al., 2002). The final diameter of the canals crucially depends on the endolymphatic formation. In *Foxi1* null mice there is a deregulation of endolymph formation and the canal diameter is progressively enlarged due to endolymphatic hydrops (Hulander et al., 2003).

Thus, ear defects generated by the patterning genes may actually be mediated through their modification of FGF's and/ or FGFR's expression. Unfortunately, in virtually all patterning mutants analyzed to date, only *Fgf8* expression has been analyzed (Ladher et al., 2005; Pauley et al., 2003; Pirvola et al., 2000; Wright and Mansour, 2003b). Expression data are needed in other morphogenetic mutants to verify our idea of Fgfs are a crucial node in ear morphogenesis.

4.3. The horizontal canal is embedded into canal formation but requires recruitment of novel genes

The essential role for FGFs and BMPs for all canal formation is supplemented by an additional set of genes that specifically affect the formation of the horizontal canal (Chang et al., 2004b). Among those genes, *Otx1* is unique in that its evolutionary history has been detailed and it appears that the function of *Otx1* in horizontal canal formation (Fig. 6) is linked to the newly acquired function of the paralogue of the ancestral orthodenticle/ocelli less gene, *Otx1*. Notably, while *Otx1* and *Otx2* can functionally replace each other in the forebrain, the function of *Otx1* in horizontal canal formation can not be replaced by *Otx2*

(Acampora et al., 2005; Cantos et al., 2000; Morsli et al., 1999). Interestingly, it appears that formation of the horizontal canal crista and the sensory neurons is independent of horizontal canal formation as an initial patch of sensory epithelium forms (Cantos et al., 2000) and later additional patches of hair cells scattered in the ear or associated with other epithelia have been reported (Fritzsch et al., 2001). Most importantly, these cells are all innervated by sensory neurons and their projection pattern is reminiscent of that reaching the horizontal canal of wildtype mice or the dorsal macula of lampreys.

Combined, these data suggest that evolution of the horizontal canal system may have occurred in two phases. In a first phase the sensory hair cells and sensory neurons formed through additional growth and splitting from other sensory epithelia. In a second phase the sensory epithelium became specifically associated with the horizontal canal after morphogenesis of the horizontal canal had evolved through co-option of *Otx1* and other genes (Cantos et al., 2000; Chang et al., 2004b). Our data suggest that *Foxg1* may be at least one gene that is crucial for sensory neuron and hair cell formation of the horizontal crista (Pauley et al., submitted for publication). Combined these data highlight crucial steps in morphogenesis, histogenesis and neurogenesis of the canal system in general and offer fresh insights into the development and evolution of the horizontal canal system.

5. Conclusion

An understanding of the molecular mechanisms causing histogenesis and morphogenesis of the vertebrate ear and its evolution is now within reach. Previous studies have focused either on the formation of cell lineages associated with the sensory epithelium, sensory neurons or on sensory epithelial morphogenesis or on ear morphogenesis. Unfortunately, while much has been learned on the morphogenesis aspect of vestibular ear formation, the neurosensory aspect has received less attention, in part owing to the more complex techniques needed to investigate this aspect. Simply completing this latter aspect in already existing mutations with known morphogenetic defects will likely expand our insights into the molecular basis of ear morphogenesis by adding the integration of neurosensory development into the functionally crucial morphogenesis of the ear. Without such integrated perspective, analysis of morphogenetic defects in mutants will suffer from a lack of understanding in the neurosensory aspect that are needed for evolutionary inferences on the significance of such changes.

Mutational analyses of the transcription and diffusible growth factors have greatly assisted in the identification of many of the genes involved in ear development. Clearly, these two processes are highly integrated. The current recognition that Choanoflagellates may have molecular components of mechanosensors should provide for the identification of more ancestral genes involved in formation and function of apical specialization beyond rare myosins. Establishing the gene network for mechanosensory cell development will enhance our understanding of the incorporation of these gene modules into the formation of the complex three dimensional vertebrate ear. Although there is a wide diversity of ear morphology, it is becoming evident that the FGF/FGFR functions as a common constraining bottleneck in ear morphogenesis and may conserve the associated gene networks as they must tie into the expression regulation of FGF/FGFRs as well as the gene network necessary to develop hair cells and neurons. Given the speed of progress within the decade since publication of the first comprehensive reviews of molecular biology of ear development (Fritzsch et al., 1998; Torres and Giraldez, 1998), we can reasonably expect that the next decade will bring us closer to resolving the molecular mechanisms of ear morphogenesis down to the cellular level, a prerequisite for the treatment of patients suffering from congenital malformations in the auditory and vestibular system.

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Fig. 1.

The taxonomic relationships of metazoa and the evolution of apical sensory cell specializations thought to be involved in mechanosensation is depicted. Even single cell organisms like choanoflagellates have some mechanosensation and some amoeba can perceive the beating of cilia of their prey. Whether this is molecularly identical to the still unknown mechanosensory channels and associated molecules of triploblasts is unknown. Orthologues of structural genes relevant for mechanosensation or for development of polarity such as actin, tubulin, rare myosins, cadherins, espin, β -catenin and Wnt genes are known in protists, diploblasts and various triploblasts and are thus ancestral to vertebrates. It is proposed that apical specializations are variations of the ancestral theme of a central cilium surrounded by microvilli found in choanoflagellates (A) as well as basic diplo- and triploblasts. Alterations in ancestral taxa transformed the single central cilium surrounded by microvilli (1,2) into the acentral kinocilium adjacent to the long stereocilia of vertebrates (3). The most derived mechanosensory cells in each lineage show dramatic variations (B–F). Some jelly fish have cilia surrounded by an asymmetric group of microvilli with variable thickness and length (B). Among mollusks, cephalopods have multiple rows of cilia interconnected by basal strands that provide directional sensitivity (indicated by an arrow in C). Flies are most derived among ecdysozoans in that they have a modified cilium with an interrupted axonem surrounded by supporting cell sheath (D). It is believed that extension and compression of the cilium provides proper stimulation (arrow in D). Among chordates, some ascidians have a somewhat asymmetric cilium (E). Cochlear hair cells lose the kinocilium in development and have tip link connections that provide directional sensitivity (arrow in F). Flies (D) and mammals (F) differ most profoundly with respect to location of the mechanotransducer system (kinocilium or stereocilia, respectively) and stimulation force direction (along the long axis of the kinocilium and perpendicular to the row of stereocilia). Establishing molecular developmental similarities and differences between fly and mammalian mechanosensory organs will therefore directly test the presumed ancestry of mechanosensory cells in triploblasts. Modified after (Arkett et al., 1988; Budelmann, 1989;

Burighel et al., 2003; Fritzsche and Beisel, 2004; Jorgensen, 1989; Steenkamp et al., 2006; Todi et al., 2004).

Evolution and Development of bHLH genes and mechanosensory cells/neurons

Fig. 2.

The evolution of the *atonal* and *neurogenin* families of bHLH genes (A) and the evolution of the mechanosensory cells and their associated neurons that require those family members for cellular development (B) is shown. Note that *atonal* and *achaete/scute* family evolved already in coelenterates. However, the *neurogenin* family may have evolved only in triploblasts. Evolution of a pair of cells (a secondary mechanosensory cell without an axon and a mechanosensory neuron connecting the cell to the brain) evolved out of a primary mechanosensory cell (with an axon) only after the *neurogenin* family had evolved. Whether *atonal* family members are always associated with mechanosensory cells in triploblasts and whether neurons associated with mechanosensory cells in other triploblasts require *neurogenin* for development is unknown. Modified after (Fritzsche and Beisel, 2004; Furlong and Graham, 2005).

Fig. 3.

Choanoflagellates have a central kinocilium surrounded by a complex set of interconnected microvilli for food capturing. These protists likely use bHLH genes in development and can build extracellular hard tissue shells (left). Jellyfish have distinct sensory and muscle cells and use several bHLH genes for histogenesis. Multiplication of bHLH genes and generation of novel bHLH gene families such as the neurogenin family allowed the generation of an increased cellular diversity. Vertebrates have further increased the number of bHLH genes per family and have evolved a specialized hair cell and a unique set of neurons that connects the information gathered by mechanosensors to the CNS. The increased number of bHLH family members gave vertebrates a larger repertoire to differentiate distinct sets of neurons in the increasingly larger brain for sensory integration and differential motor output. Modified after (Burighel et al., 2003; Mackie, 1990; Seipel et al., 2004). Choanoflagellate image is courtesy of B.S. Leadbeater.

A. Evolution of the vertebrate ear

B. Development of sensory patches of a salamander

Fig. 4.

Crucial steps in vertebrate ear evolution (A) and development (B) are depicted. It is assumed that the vertebrate hair cells with afferents and efferents co-evolved with the ear (insert) some 600 Million years ago. Note that the ears of the three depicted vertebrate species differ in the number of canal cristae (hagfish has two, coelacanth (*Latimeria*) and mouse have three), number of vestibular organs (hagfish has one [common macula], coelacanth has three [utricle, U; saccule, S; lagena, L] and mouse has two [utricle, U; saccule, S]) and number of organs near perilymphatic ducts (none in hagfish, two [basilar papilla, BP; papilla neglecta, PN] in coelacanth and one [organ of Corti of the cochlea, C] in mouse). Major morphological evolutionary changes are the addition of a horizontal canal in gnathostomes and the transformation of the simple doughnut ear into several recesses containing the utricle, saccule, lagena and cochlea. It is suggested that the evolution of up to nine sensory organs of the vertebrate ear (A) comes about through ontogenetic segregation of a single primordium into multiple sensory patches (B). After segregation, each sensory patch differentiates along a unique trajectory to form adult epithelia that perceive discrete aspects of the mechanical stimulation that reaches the ear (A). Development (B) therefore recapitulates the evolutionary segregation and differentiation of various epithelia from a common precursor. Integrated into this differentiation is the organization of different polarities of hair cells (arrows in B) that can be opposing (utricle, saccule, lagena) or of one polarity (canal cristae). Note that the polarity of hair cells in the cristae is similar in anterior and posterior crista (away from the gravistatic organs) whereas the horizontal crista is polarized toward the gravistatic organs. How the ancestral molecular pathway to set up

cellular polarity in the various sensory epithelia has been modified remains unclear. Modified after (Fritzsche and Beisel, 2004; Fritzsche et al., 2002).

Fig. 5.

The development of the human ear (top row: A, B, C) is shown as well as ear defects published in various null mutations. Note that the growth of the otocyst is paralleled by the sculpting of the canals and the formation of neurons innervating specific sensory epithelia. Bottom rows, left column: *Gata3*, *Eya1*, and *Fgf3/10* are essential for the growth and overall development of an ear past the otocyst. These genes are expressed in the ear and promote their effect by interacting with other genes expressed in the ear and may change their expression in null mutants (shown here for *Gata3*). Second column from left: 'Cochlear genes' result primarily in a cochlear or ventral otocyst defect. These genes seem to affect ventral patterning either through a diffusion gradient (*Shh*) or by being differentially expressed (*Pax2*). Mutations in these genes form only a short cochlear duct (CD). The vestibular system forms at least the vertical canals. Right two columns: 'Vestibular genes' cause primarily a vestibular phenotype. Such genes may exert their effect from their expression in the brain. Genes that fall into this category are *Wnt1/3a* and possibly *Gbx2*. Other genes expressed in the ear such as *Hmx2/3* causes almost as much defect as *Wnt1/3a* double null mice. The phenotype of such mutants is an elongated sack that may be composed of the endolymphatic duct (ED) and parts of the cochlea (C). Other genes exert their effects via expressions in the otocyst. Interestingly, despite different expression

patterns, *Fgf10* has a phenotype somewhat similar to *Gbx2* null but has in addition lost the horizontal canal. *Otx1* null mice lack only the horizontal canal and show a confluence of utricle (U) and saccule (S). The caudal expansion of the utricular epithelium has been interpreted to be the horizontal crista (HC). *Fgfr2b* null mice develop a sack-like ear somewhat similar to *Hmx2/3* and *Wnt1/3a* null mice. *Fgfr2b* and *Fgf10* are likely to exert their effect directly in the ear as this is their main expression area. Compiled after (Bok et al., 2005; Burton et al., 2004; Karis et al., 2001; Lin et al., 2005; Pauley et al., 2003; Riccomagno et al., 2002; Riccomagno et al., 2005; Wang and Lufkin, 2005).

Fig. 6.

This cartoon shows the known and suspected interactions of some transcription factors in ear development. Sonic hedgehog (*Shh*) forms a ventral to dorsal gradient throughout the hindbrain and spinal cord, *BMP4*, *Gli3* and *Wnt1/3a* form a dorsal to ventral gradient (black arrows in the hindbrain slices). These gradients may be locally varied by the expression of the *Hox* genes in each rhombomere (r5 and r6). Interactions of the brain with the forming otocyst (indicated by black arrows) regulate the expression of various ear morphogenetic genes such as *Fgf3/10*, *Fgfr2b*, *Hmx2/3*, *Gata3*, *Pax2* and *Neurog1*. Removal of dorsal specifying genes in the hindbrain or otocyst (*Wnt1/3a*, *Hmx2/3*, *Fgfr2b*) results in loss of vestibular development. Likewise, removing ventrally specifying genes (*Shh*, *Pax2*) will reduce ventral otocyst differentiation. It is proposed that most of these factors act through the regulation of growth promoting factors such as *Fgf10*, *Fgf3* and their cognate receptor *Fgfr2b*. The importance of *Fgf3/10* is underscored by the near complete loss of otocyst differentiation in double null mutants, far more profound than any other combination of genes thus far reported for mammals. Modified after (Fritzsch et al., 1998; Riccomagno et al., 2005; Xiang et al., 2003).

Table 1
Examples of histogenetic and morphogenetic defects of genes conserved between flies and mammals

	Mouse	Fly (orthologue or paralogue)	Type	Defects:	Ref:
Genes involved in histogenesis	Atoh 1	atonal	bHLH	No hair cell differentiation in the null	(Bermingham et al., 1999; Fritzsche et al., 2005a)
	Pou4f3, Brn3c	acj6	POU domain transcription factor	Hair cells die	(Clough et al., 2004)
	Barhl1/BarH-like 1, MBH2	B-H1/BarH1	homeodomain	Hair cells die at P30 in Barhl1 null	(Li et al., 2002)
	Gfi1	senseless	zinc finger	Hair cells die	(Wallis et al., 2003)
	Hes/hairy and enhancer of split	hairy	HLH	Supernumerary hair cells	(Zine et al., 2001)
	Foxg1, (BF-1)	sloppy	forkhead	Supernumerary hair cells	(Pauley et al., submitted for publication)
	Neurog1	conserved in Amniota	bHLH	No sensory neurons in the null	(Ma et al., 2000; Ma et al., 1998)
	Neurod1	conserved in Bilateria	bHLH	Sensory neurons die	(Kim et al., 2001; Liu et al., 2000)
	Fgfr	bt/breathless	tyrosine kinase receptor	Ear development arrested at otocyst stage	(Pirvola et al., 2000)
	Gata3	grn/grain	zinc finger	Ear development arrested at otocyst stage	(Karis et al., 2001)
Genes involved in morphogenesis	Eya	eya/eyes absent, clift	transcription coactivator	Regulates expression of Dach1, Tbx, Neurog, and Neurod in the otocyst	(Zou et al., 2004)
	Six1/sine oculis-related homeobox 1 homolog	so/sine oculis	homeodomain	Ear development arrested at otocyst stage	(Zou et al., 2004)
	Fgf	bnl/branchless	fibroblast growth factor	Mild to profound morphogenetic defects in some mutants	(Pauley et al., 2003; Wright and Mansour, 2003a),
	Bmp	dpp/decapentaplegic	transforming growth factor beta family	Vestibular defect	(Chang et al., 2004b; Morsli et al., 1999)
	Wnt	wg/wingless	wnt factor	Vestibular defects in nulls	(Riccomagno et al., 2005)
	Hmx	Hmx/H6-like homeobox	homeodomain	Vestibular defects in nulls	(Bober et al., 2003; Wang et al., 2001, 2004)
	Gbx2/ gastrulation brain homeo box 2	unpg/unplugged	homeodomain	Vestibular defects in nulls	(Lin et al., 2005)
	Otx	oc/ocelliless, otd/ orthodenticle	transcription factor	No horizontal canal in Otx1 null	(Fritzsche et al., 2001; Morsli et al., 1999)
	Pax2	Dpax 2, sparkling	transcription factor	Cochlear defects in nulls, regulates frizzled related protein 2	(Burton et al., 2004)

Mouse	Fly (orthologue or paralogue)	Type	Defects:	Ref:
Pax3	prd/paired	transcription factor	Ear defects/ Waardenburg's Syndrome 1	(Deol, 1983)
Shh/sonic hedgehog	hh/hedgehog	secreted factor	Cochlear defects in the null, regulates Pax2	(Riccomagno et al., 2002)