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GATA3 AND GATA2 IN INNER EAR DEVELOPMENT

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LIST OF ABBREVIATIONS

A/P – antero-posterior
BDNF – brain-derived neurotrophic factor
bHLH – basic Helix-Loop-Helix
cFgf10 – chicken fibroblast growth factor 10
cGata2 – chicken Gata2
cGata3 – chicken Gata3
Cx – connexin
D/V – dorso-ventral
E – embryonic day
ECM – extracellular matrix
Fgf – fibroblast growth factor
FOG – friend of Gata
Gj – gap junction
HH – stages according to Hamburger and Hamilton
IHC – inner hair cells
Jag1 – Jagged1
Jag2 – Jagged2
M/L – medio-lateral
mFgf10 – mouse fibroblast growth factor 10
mGata2 – mouse Gata2
mGata3 – mouse Gata3
Ngn1 – neurogenin 1
NT3 – neurotrophin 3
OHC – outer hair cells
Pax – paired-box
r – rhombomere
Spp1 – secreted phosphoprotein 1
Th – T helper cell
Ush – U-shaped
wt – wild type
ZF – zinc finger

Names of genes are italicised; proteins are in regular font.

ABSTRACT

The inner ear develops from the otic placode that arises adjacent to the caudal hindbrain. The otic placode first invaginates, forming an otic cup, and thereafter closes to form the otic vesicle, which undergoes a complex morphogenetic process to form the membranous labyrinth that comprises a dorsal vestibular apparatus for balance and a ventrally positioned auditory structure. The sensory functions of inner ear are carried out by specialized hair cells that are organized into distinct sensory organs. The molecular background of inner ear morphogenesis and hair cell differentiation is relatively little understood. The aim of this work was to gain a better insight into the roles of two transcription factors Gata3 and Gata2 in inner ear morphogenesis and sensory cell development using gene targeted mouse mutants as tools. In addition, the expression of these genes was compared in mouse and chicken embryos in order to obtain more information about their mutual relationship and conservation of their roles among vertebrates.

The absence of *Gata3* expression led to very early defects. The otic cup morphology was aberrant, and the formed vesicle was reduced or divided in two parts. A large-scale expression analysis suggested that alterations in cell adhesion and motility may underlie the abnormal early otic morphology in *Gata3–/–* embryos. In addition to the early morphogenetic abnormalities and reduced expression of many dorsal otic genes, loss of *Fgf10* expression from *Gata3–/–* otic region was likely to contribute to the complete absence of semicircular ducts. Transactivation studies in cell cultures suggested that *Fgf10* could actually be the first direct target gene for Gata3. In spite of the serious problems in early morphogenesis, the axial polarity of the vesicles was not altered. The general sensory fate was established in *Gata3–/–* otic epithelium, and vestibular hair cells developed. Interestingly, however, the cochlear sensory differentiation was abolished, and no postmitotic auditory hair cells could be detected.

In sharp contrast to *Gata3–/–*, no early otic defects were observed in *Gata2–/–* embryos. Instead, conditionally inactivated *Gata2* embryos suffered from a relatively late growth defect in the semicircular ducts. The lack of early defects could be due to redundant functions of Gata3, which was normally expressed in *Gata2–/–* ears.

In both mouse and chicken embryos, the expression of *Gata3* preceded *Gata2* and the two genes presented a remarkable overlap during early stages. Later, *Gata3* became prominent in cochlear sensory epithelium and *Gata2* in vestibular nonsensory regions in both species indicating high conservation of roles. Major differences between mouse and chicken *Gata3* expression were observed only during early otic development providing indications of evolutionary divergence of the molecular mechanisms involved in placode morphogenesis of birds and mammals.

1. LITERATURE OVERVIEW

1.1. Inner ear – sensory structure for balance and hearing

The inner ear is organised into a complex three-dimensional membranous labyrinth tightly enclosed into a bony capsule. It comprises a dorsal vestibular apparatus for gravity detection and balance and a ventrally positioned auditory system for hearing (Figure 1). Both structures contain distinct sensory epithelia responsible for transforming sound waves or angular and linear movements into electrical signals and conveying them to the brainstem in connection with associated neurons of the vestibulo-cochlear ganglion (reviewed in Riley and Phillips, 2003; Barald and Kelley, 2004).

The dorsalmost part of the vestibular apparatus includes three orthogonally arranged semicircular ducts (superior, posterior, and lateral) that are responsible for detecting angular head movements (reviewed in Highstein *et al*., 2005). This part is highly conserved between vertebrate classes (Figure 1), differing only in details (reviewed in Riley and Phillips, 2003). However, in contrast to jawed vertebrates, the recent jawless organisms, the lampreys and hagfish, lack the lateral canal system, as observed in the jawless vertebrate fossils (reviewed in Fritzsch *et al.*, 2006a; Mazan *et al.*, 2000).

Vestibular apparatus is also responsible for detecting gravity and linear acceleration. These functions are carried out by special chambers among which the utricle is highly conserved in vertebrates. Similar functions are covered also by the saccule in mammals and birds.

In contrast to vestibule, the auditory apparatus differs to a great extent between vertebrate classes (Figure 1). While mammals and birds form a cochlear duct for hearing, fishes and amphibians have developed their saccule into an auditory structure and do not possess a morphological counterpart of cochlea. Lagena is another auditory organ in fish, and it serves for vestibular functions in birds and amphibians, whereas it is completely missing in mammals. Frogs possess two additional auditory organs called amphibian and basilar papillae; the latter is thought to be related to cochlea (reviewed in Riley and Phillips, 2003, Zakir *et al.*, 2003 and references within).

The membranous labyrinth is filled with endolymph, the pressure of which is controlled by the dorsally extending endolymphatic duct and sac (Salt and Rask-Andersen, 2004). Endolymph is a liquid of unusual ionic balance (high K^+, low Na^+). It is formed by the stria vascularis in mammals and the tegmentum vaculosum in birds, the epithelium locating in the lateral wall of the cochlear duct and by dark cells adjacent to the cristae ampullaris (reviewed in Torres and Giraldez, 1998; Hara *et al*., 2002). The space between the membranous labyrinth and the otic capsule is filled with perilymph, the content of which (high Na^+, low K^+) resembles that of other body liquids. Ionic differences

between endolymph and perilymph are essential for the sensory functions of inner ear (reviewed below).

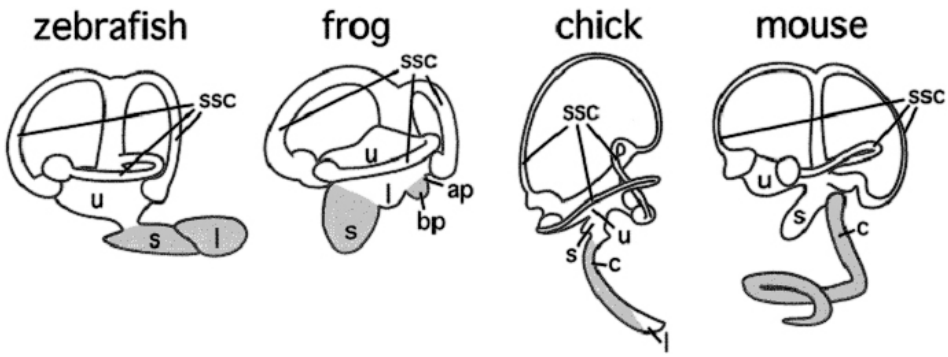

Figure 1. The inner ears of different vertebrate class representatives. ap, amphibian papilla; bp, basilar papilla; c, cochlea; l, lagena; s, saccule; ssc, semicircular canal (duct); u, utricle. Grey areas mark auditory regions. The endolymphatic duct is not shown (From Riley and Phillips, 2003, with permission from Elsevier).

1.1.1. Sensory organs

The sensory functions of inner ear are carried out by specialized hair cells that are organized into distinct sensory organs. These special cells serve as mechanoreceptors by converting the mechanical movement of their polarized hair bundles into neuronal stimuli. The hair bundle consists of an array of stereocilia (actin-rich microvilli), which are arranged in rows that decrease in height further away from the single kinocilium – the true cilium containing a 9+2 arrangement of microtubules (Shin *et al.,* 2005; reviewed in Kelley, 2006).

The hair bundle is directionally sensitive, so that its deflection towards the tallest row of stereocilia causes the opening of apical K^+ channels of the stereocilia and the kinocilium, which are located in potassium-rich endolymph, and the hair cells become depolarized resulting subsequently in Ca^{2+} canal opening. Ca^{2+} entrance into the cell causes an increase of neurotransmitter (aspartate or glutamate) release from the base of the cell. The signals are transduced into brainstem by afferent neurons, the cell bodies of which lie in vestibular and cochlear ganglia. Efferent innervation provides also feedback to sensory epithelium. Stereocilia deflection towards the shortest row leads to the closure of apical K^+ channels and opening of the K^+ channels in the basolateral portion of the hair cells, the latter being surrounded by potassium-low perilymph. This situation leads to K^+ outflux from a hair cell, and its subsequent hyperpolarization is accompanied with the closure of Ca^{2+} channels and decrease of neurotransmitter release (Figure 2).

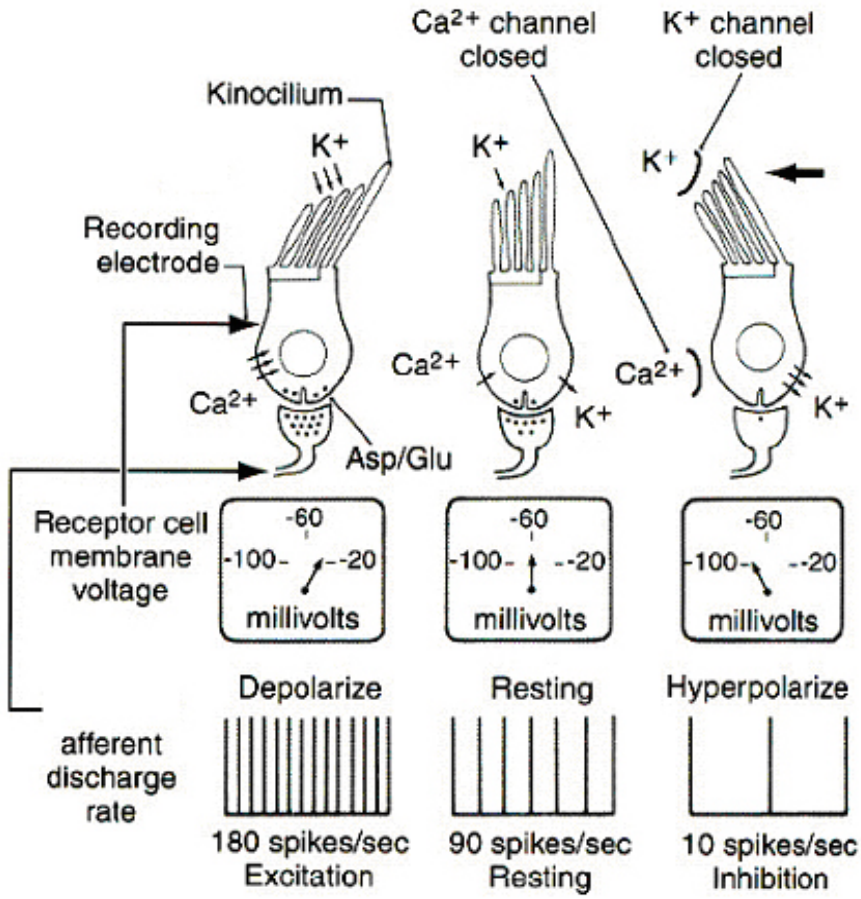

Figure 2. Physiological responses of hair cells and their afferent fibres. Asp, aspartate; Glu, glutamate (according to Dickman, 2006).

In each sensory organ the hair cells are surrounded by supporting cells (Figure 3), the functions of which are less understood. One of the functions of the supporting cells is to secrete the extracellular matrices that lie above the sensory epithelia and influence the cilia bending. In contrast to vestibular sensory epithelia (Figure 3A,B), which contain only one type of supporting cells, different types of supporting cells exist in mammalian cochlea (Figure 3D) (Kiernan *et al*., 2002).

Three to nine distinct sensory organs are located in the inner ear, depending on the species of vertebrates. Mammalian inner ear comprises six sensory organs: cristae at the base of each semicircular duct, maculae utricle and saccule as well as organ of Corti locating along the cochlear duct (Morsli *et al.*, 1998). In birds the auditory sensory epithelium located in the cochlear duct is called

basilar papilla, and the vestibular apparatus contains additionally maculae neglecta and lagena (Wu and Oh, 1996). The structure and exact position of the sensory organs depend on their particular task. In general, three types of sensory organs exist:

i) The cristae are housed in the enlarged part of the semicircular ducts (ampullae) in a way that the ciliary bundles of the hair cells located in the cupular extracellular matrix (ECM) can detect head movements in any direction (reviewed in Beisel *et al.*, 2005) (Figure 3A).
ii) The maculae are responsible for detecting linear acceleration and gravity. Their sensory cells are overlaid with a dense otolithic membrane, which provides stronger inertia to stereociliary bundles (Lundberg *et al.*, 2006). The hair cell polarity changes about 180 degrees in the middle of the maculae, in the striolar region, either towards the striola (in saccule) or away (utricle). The striola curves through the macula, and, as a result, the hair cells are polarized in several directions. It makes the macular hair cells directionally sensitive to a wide variety of head positions and linear movements (Denman-Johnson and Forge, 1999; Jaeger *et al.*, 2002) (Figure 3B,C).
iii) Auditory sensory organs differ largely among vertebrates (reviewed in Riley and Phillips, 2003). It has been suggested that auditory epithelia of the different groups of amniotes (mammals, birds, lizards) evolved independently from each other (reviewed in Manley and Köppl, 1998). In all of these three groups the original papilla elongated and the hair cells separated into two different populations. In most species hair cells are covered by an acellular tectorial membrane. Mammals and birds have one group of hair cells that is specialized for sensory reception (inner hair cells in mammals and tall hair cells in birds) and another group specialized for sound amplification (outer hair cells in mammals and short hair cells in birds). In contrast to birds, the mammalian auditory hair cells are organised into four longitudinal arrays along the cochlear duct – one row of inner hair cells (IHC) and three rows of outer hair cells (OHC) (Figure 3D). In birds and mammals the auditory sensory epithelium is located over a movable membrane, the basilar membrane (BM). Distinct regions of the BM vibrate in response to sound waves with some frequency selectivity. Unlike other animal groups, in mammals the frequency selectivity is amplified due to the motor activity of outer hair cells (reviewed in Manley, 2000; Dallos *et al.*, 2006).

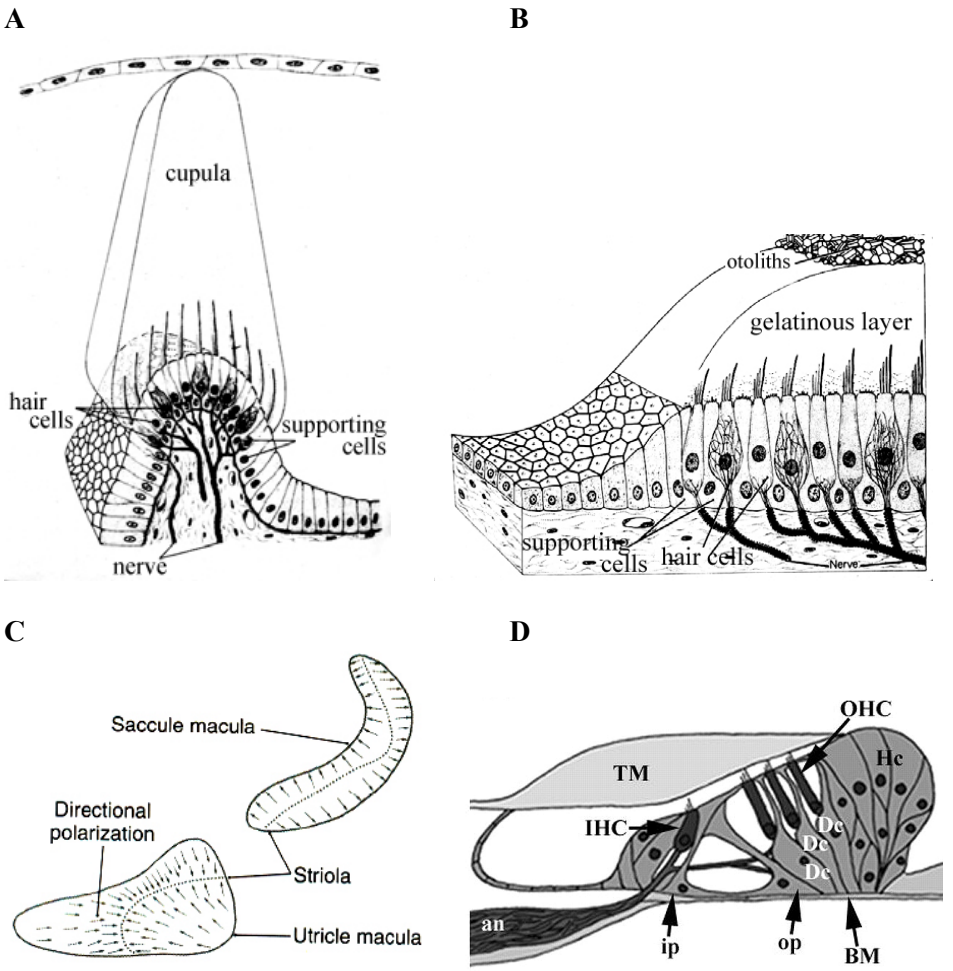

Figure 3. The structure of sensory organs in mammals. A – crista; B – macula; C – hair cell organization in the maculae, arrows indicate hair cell polarity; D – cross-section through the organ of Corti. an, afferent nerve; BM, basilar membrane; Dc, Deiter's cell; IHC, inner hair cell; He, Hensen's cells; ip, inner pillar cell; OHC, outer hair cell; op, outer pillar cell; TM, tectorial membrane (according to Dickman, 2006; Forge and Wright, 2002; Junqueira and Carneiro 2005, with the permission from McGraw-Hill).

1.2. Sensorineural hearing disorders and regeneration of sensory hair cells

Approximately 1/3 of the human population over the age of 65 exhibit considerable hearing loss (Li *et al.*, 2003). About 60 per cent of the congenital cases are caused by genetic factors (Piatto *et al.*, 2005). In more than 80 per cent of the cases hearing impairment in humans is a direct consequence of damaged inner ear sensory hair cells and associated auditory neurons (Li *et al*., 2003). New hair cells form only during a limited period of mammalian embryonic development, and therefore their loss causes irreversible hearing/equilibrium disorders. In contrast to mammals, hair cells regenerate after damage or undergo continuous replacement in most non-mammalian vertebrates during the whole life span (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Stone and Rubel, 2000; Bermingham-McDonogh and Rubel, 2003). Understanding the molecular pathways and the underlying mechanisms for this difference may provide a valuable key when designing new therapies for human deafness.

1.2.1. Molecular background of sensorineural deafness

Around 40 genes are known to be involved in non-syndromic hearing disorders (Petersen and Willems, 2006). Mutations in several of these genes affect directly sensory hair cells, whereas other mutations cause alterations in surrounding cells leading as a secondary consequence to the impairment of hair cell functions.

Hair cell functioning depends on the precise organization and renewal of its stereociliary bundles. An active actin treadmill has been shown to maintain each stereocilium (Schneider *et al.*, 2002; Rzadzinska *et al.*, 2004). Accordingly, several hearing/balance disorders, including various forms of Usher's Syndrome, are caused by mutations in genes encoding proteins involved in the formation and maintenance of the architecture of hair cell stereocilia (reviewed in Hawkins and Lowett, 2004).

In addition to the precise cytoarchitecture, the exact cochlear homeostasis is absolutely essential for auditory sensory hair cell functions (see 1.1.1.). Stria vascularis secretes an unusual extracellular fluid, the endolymph, and generates the largest transepithelial voltage in the whole organism. This endocochlear potential is in principle a K^+ equilibrium potential and is generated by the K^+ channel KCNJ10 located in the intermediate cells of stria vascularis (Marcus *et al.,* 2002). Not surprisingly, mutations in several genes that encode K^+ channel proteins cause severe hearing loss (reviewed in Jentsch, 2000).

Gap junctions are channels between neighbouring cells and are involved in mediating ion flux. They consist of two homo- or heterohexameric hemichannels, which comprise connexin (Cx) proteins, also called gap junction (Gj)

membrane proteins. Around 20 Cx encoding genes have been found in humans and mice and almost all cells in mammals are interconnected by gap junctions. Several *connexin* genes are expressed in the inner ear, two of which, *connexin26 (Gjb2)* and *connexin30 (Gjb6)*, are coexpressed in supporting cells and fibrocytes and are considered to be critical in K^+ uptake from perilymph (reviewed in Willecke *et al.*, 2002; Forge *et al*., 2003; reviewed in Wangemann, 2006). Mice with mutated *Gjb2* are deaf and also mice deficient in *Gjb6* exhibit a severe hearing loss. Remarkably, the mutations in *GJB2* are the most common cause of human hereditary deafness (reviewed in Petersen and Willems, 2006). Similarly, the mutations in *GJB6* lead to common hearing loss in humans (Del Castillo *et al.*, 2002).

The tectorial membrane produced by cochlear supporting cells contacts the stereocilia bundles of the hair cells and plays an essential role in auditory transduction. Particularly, auditory hair cells and the above located tectorial membrane move from different pivot points because of cochlear vibration, causing a sharing motion that bends the hair cell stereocilia appropriately (Steel *et al.*, 2000). Severe hearing problems are caused by alterations in genes encoding proteins involved in tectorial membrane composition, for example Collagen XI (α2 chain) and Alpha-tectorine (Verhoeven *et al.*, 1998; McGuirt, 1999; Pfister *et al.*, 2004)**.**

Alterations in several transcription factor-encoding genes cause syndromic deafness. For example, the Renal-coloboma syndrome is caused by mutations in paired box homeodomain gene, *PAX2*. The patients suffer from optic nerve colobomas and various degrees of kidney abnormalities; additionally, sensoryneural hearing loss has been described in several patients (Burton *et al*., 2004). Mutations in another paired box gene *PAX3* cause the Waardenburg syndrome characterized by sensorineural hearing loss associated with pigmentary anomalies (Karaman and Aliagaoglu, 2006).

The most common syndromal form of deafness is the Pendred's syndrome being a consequence of alterations in the *SLC26A4* gene. It is characterized by sensorineural hearing loss, variable vestibular dysfunction and thyroid abnormalities (reviewed in Glaser, 2003). *SLC26A4* encodes a transmembrane protein pendrin, which functions as a transporter of Cl^- and I^- and thus controls the fluid homeostasis in the membranous labyrinth (Scott *et al.*, 1999). It is expressed in various cell types that are important for endolymph resorption, such as endolymphatic duct and sac epithelium and spiral prominence cells and/or external sulcus root cells in the cochlear duct (Everett *et al*., 1999).

1.2.2. Induction of hair cell regeneration – a possibility for deafness therapy?

Hair cells and supporting cells originate from common progenitors during development (Fekete *et al.*, 1998). In adult birds hair cell degeneration in the cochlea triggers neighbouring supporting cells to divide and give rise to both new hair cells and supporting cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). A small portion of newly formed hair cells, however, develops from supporting cells without preceding cell division (Roberson *et al*., 2004).

Unlike birds, the precursors of hair and supporting cells in mammalian cochleae undergo terminal mitosis during embryogenesis and are unable to re-enter the cell cycle after the damage of their neighbours. Thus, one key to hearing loss therapies may lie in overcoming the cell cycle arrest, which in sensory progenitors is mainly regulated by $p27^{Kip1}$ and Rb1. Later, supporting cell proliferation is negatively regulated via $p27^{Kip1}$ and Rb1, whereas hair cell quiescence depends largely on Rb1 alone (Chen and Segil, 1999; Löwenheim *et al.*, 1999; Mantela *et al.*, 2005; Sage *et al.*, 2005). Indeed, mice with targeted $p27^{Kip1}$ develop supernumerary hair and supporting cells, and the latter continue to proliferate also in postnatal cochlea (Chen and Segil, 1999). *Rb1* knockouts exhibit supernumerary sensory cell progenitors that develop into hair and supporting cells (Sage *et al*., 2006). During later stages the lack of Rb1 has been shown to cause the production of excess auditory hair cells via hair cell proliferation. However, these cells exhibit many pathological features (Mantela *et al.*, 2005). In addition, recent work has shown that supporting cells isolated from adult mouse cochleae were able to downregulate $p27^{Kip1}$ expression, divide and trans-differentiate into hair cells when cultivated together with embryonic periotic mesenchyme (White *et al.*, 2006). Taken together, understanding the negative cell cycle regulation of hair cells and supporting cells is currently one of the most promising ways to find new solutions for therapies of deafness caused by hair cell loss.

Another promising factor for deafness therapy is the basic Helix-Loop-Helix (bHLH) domain containing proneural transcription factor Math1, an essential factor in hair cell differentiation (see 1.3.4.3.). Overexpression of *Math1* in neonatal rat cochleae has been shown to induce new hair cell production (Zheng and Gao, 2000). Moreover, adult deafened guinea pigs restored their hearing capability considerably after the delivery of Math1 to non-sensory cells **(**Izumikawa *et al.*, 2005).

1.3. Inner ear development

Most inner ear cells and tissues, as well as the neurons in the vestibulo-acoustic ganglion, develop from a simple ectodermal placode. Only the secretory pigment cells of stria vascularis are neural crest derived (reviewed in Torres and Giraldez, 1998).

1.3.1. Induction

The first classical studies on otic induction showed that otic competence in the early stages of development is a rather widespread property of ectoderm, which becomes more restricted with age. Induction was understood to be a gradual process that occurs during a relatively long span of time and signals both from the hindbrain and the mesoderm are likely to be necessary (reviewed in Groves and Bronner-Fraser, 2000; Riley and Phillips, 2003). More recently, gene-targeting studies have not been able to identify any single mutation that would lead to otic induction failure. Thus, it has been proposed that at least two synergically acting signals are needed to induce otic placode in a competent non-neural ectoderm: one from the cephalic paraxial mesoderm and the other from the neural ectoderm (reviewed in Noramly and Grainger, 2002).

1.3.1.1. Members of Fgf and Wnt families

Members of the fibroblast growth factor (Fgf) family have been shown to play a major role in otic induction across vertebrates. *Fgf3* is expressed in the caudal hindbrain of all main vertebrate model organisms (zebrafish, *Xenopus*, chicken, mouse) just prior to otic placode induction, making it a good candidate for an ear inducer. In the mouse, ectoderm-derived Fgf3 together with mesoderm-derived Fgf10 are the main known otic inducers (Alvarez *et al.*, 2003; Wright and Mansour, 2003). Additionally, *Fgf8*, being expressed in or next to tissues required for otic induction, as well as preplacodal ectoderm, has also been considered to play an important role in otic induction (Ladher *et al.*, 2005). While the mutation in one of these family members does not disturb otic induction in the mouse, otic induction in *Fgf3/Fgf10* and *Fgf3/Fgf8* double mutants is severely affected. However, otic signs were sometimes still recorded in these mutants indicating that additional inducing molecules remain to be discovered (Alvarez *et al.*, 2003; Wright and Mansour, 2003). Mice with targeted *Fgfr2(IIIb)*, the gene encoding the main receptor for Fgf3 and Fgf10, develop only slightly reduced otic vesicles that are much less affected than those of *Fgf3/Fgf10* double knockout ears (Pirvola *et al.*, 2000), which indicate that other receptors are involved in the inductive process as well. The importance of Fgf family members in otic induction is supported also by the fact that the overexpression of *Fgf10,* and to a lesser extent of *Fgf3,* in the

caudal hindbrain induces ectopic otic vesicles in the mouse (Alvarez *et al.*, 2003). Similarly, the misexpression of *Fgf3* in the chicken and *Xenopus* induces otic placodes in ectopic locations (Lombardo *et al.*, 1998; Vendrell *et al.*, 2000).

Studies in the chicken have shown the importance of Wnt factors (Wnt8c) in otic induction (Ladher *et al*., 2000). Recently, Ladher and his colleagues (2005) showed also the necessity of endoderm in otic induction. Particularly, endodermal Fgf8 induces the expression of *Fgf19* in chicken mesoderm adjacent to the presumptive otic field that is required for initiation or maintenance of Wnt8c expression in neural ectoderm. The synergistic signalling of Fgf19 and Wnt8c is responsible for otic placode induction.

In the zebrafish, *Fgf8* is expressed in the hindbrain together with *Fgf3,* and they are redundantly required for otic induction (Raible and Brand, 2001). The elimination of these factors leads to complete failure of otic induction. In contrast to chicken, where Wnt8c is considered to be a direct inductor, its zebrafish homologue regulates the onset of *Fgf8* and *Fgf3* expression but is not directly required itself for the inductive process (Phillips *et al.*, 2004).

1.3.2. Early development: from placode to otic vesicle

The induced otic placode is an ectodermal thickening that appears laterally next to the developing hindbrain at the level of rhombomeres (r) 5 and 6 at 8–10 somite stage in mammals and birds. The otic placode first invaginates to form an otic cup and thereafter an otic vesicle (reviewed in Torres and Giraldez, 1998; Rinkwitz *et al*., 2001). Unlike mammals and birds, in fish the otic vesicle forms by cavitation instead of invagination (Barald and Kelley, 2004).

The driving forces behind the invagination process are largely unknown. However it is thought that the pressure of the surrounding tissue is important instead of active changes of the cytoskeleton, as has been shown in optic vesicle formation and in the nasal placode (reviewed in Legan and Richardson, 1997). Also, the attachment between the otic epithelium and the basal lamina of the neural tube is thought to be important during the otic invagination process in the chicken (Brown *et al*., 1998; Moro-Balbas *et al*., 2000; Visconti and Hilfer, 2002). Fate mapping studies have shown extensive cellular movements during invagination and otic cup closure. The postero-ventral region of the early otic cup gives rise to the whole lateral half of the otic vesicle, whereas the dorsal portion of the otic cup contributes mainly to the future endolymphatic region (Brigande *et al.*, 2000a). Finally, the closure of the otic vesicle needs apoptotic cell death in the cells that connect the surface ectoderm with otic epithelium (Cecconi *et al.*, 2004).

1.3.2.1. Hindbrain influences in otic vesicle formation and patterning

In addition to the role in otic induction, several mouse mutants with altered hindbrain patterning have clearly demonstrated that neural tissue has a major role in the correct formation and patterning of the otic vesicle.

In mammals and birds, the signalling from r5 appears to be most essential for correct ear development. For example, the loss of r5 in *Hoxa1* and *kreisler* (*Mafb* gene) mouse mutants leads to the formation of otic vesicles reduced in size (McKay *et al*., 1996; reviewed in Kiernan *et al*, 2002). The alteration of the otic vesicle in *Mafb* mutant is accompanied with remarkable changes in early otic epithelium patterning; the expression of dorsal genes is either lacking (*Gbx2, Wnt2b*) or is downregulated (*Dlx5*) from the dorsalmost region and the expression domain of a ventral gene (*Otx2*) is expanded dorso-medially (Choo *et al.,* 2006). On the other hand, the double knockout of retinoic acid receptors *Rara* and *Rarb* presents an enlargement of r5 and results in the formation of an enlarged otic cup and sometimes an additional ectopic otic vesicle (Dupe *et al.,* 1999).

It is likely that the hindbrain determines the exact location of the otic vesicle in different species. Notably, the anterior border of the *Hox* gene expression pattern differs between lampreys and higher vertebrates; accordingly, also the location of the otic vesicle is different (Murakami *et al.*, 2004).

Polarization of the early otic epithelium starts soon after otic placode formation and is highly accomplished by the signals emanating from the hindbrain. Rotation experiments in the chicken have shown that the axial polarity of the otic epithelium is established gradually, so that at first the antero-posterior (A/P) is fixed, then the medio-lateral (M/L), and finally the dorso-ventral (D/V) axis (Wu *et al.,* 1998).

The border between r5 and r6 divides the adjacent placode virtually into two equal halves. Thus, it makes possible that the signals emanating from these rhombomeres contribute to the A/P patterning of the otic epithelium. One of the best candidates for this kind of influence is the Eph/ephrin signalling, the members of which are expressed in a complementary manner in rhombomeres adjacent to the otic placode (Brigande *et al.*, 2000b). Interestingly, the timing of A/P patterning of sensory and non-sensory structures seems to be non-synchronous. In particular, when an almost closed chicken otic vesicle was removed and transplanted in reverse orientation to the host, the A/P patterning of the sensory structures was specified according to the donor and the non-sensory structures according to the host (reviewed in Cantos *et al.*, 2000).

The hindbrain is important also for the most plastic, D/V axis formation. Wnt family members (Wnt3a and Wnt1 in mouse) together with Bmp4 expressed in the dorsal hindbrain antagonize the ventralizing effect of Shh secreted from the notochord and the floorplate of the neural tube. The counter-gradient of these factors determines the expression domains of downstream genes that define the D/V regional identity of the otic epithelium (Riccomagno

et al., 2002; 2005; reviewed in Fritzsch *et al*., 2006a). Unlike the mouse where Shh is involved in D/V axis formation, Shh signalling determines the A/P axis in the zebrafish. However, the situation may be more complex and conservation may still exist as the A/P patterning has not been extensively studied in *Shh* mutant mice (reviewed in Riley and Phillips, 2003).

The lateral and medial compartments of early otic vesicle epithelium are distinguished by the expression domains of different genes (e.g. expression of *Pax2* is confined to the medial while *Hmx2* and *Hmx3* are confined to the lateral side of the mouse otic vesicle). However, the signals determining the M/L axis have remained largely unknown.

1.3.2.2. Compartment-boundary hypothesis

Brigande *et al.* (2000b) proposed a compartment-boundary hypothesis that considers the broad gene expression domains of the early otic epithelium as lineage-restricted functional compartments of the future inner ear. This hypothesis is supported by fate mapping studies in the chicken, which have revealed that during otic vesicle closure cells indeed do not mix across M/L boundary (Brigande *et al.*, 2000a). Furthermore, ablation of several genes, the expressions of which are regionalized in early otic epithelium, results in defects of specific ear structure(s) (reviewed in Kiernan *et al.*, 2002 and discussed below).

According to the compartment-boundary hypothesis, the gene expression borders may function as signalling centres specifying sensory organ location and inducing the endolymphatic duct outgrowth. Brigande *et al*. (2000b) demonstrated that in chickens the endolymphatic duct arises next to the M/L boundary defined by the juxtaposition of medial *Pax2*/*EphA4* and lateral *SOHo* expression domains. Also, the fact that the endolymphatic duct does not develop in apoptosis deficient *Apaf1–/–/Bcl2l–/–* mice (Cecconi *et al.*, 2004) is in agreement with this model. Particularly, the otic vesicle is not able to close without apoptosis and thus the borders of different compartments cannot meet and induce the endolymphatic duct outgrowth. The study by Brigande *et al.* (2000b) also shows that in chicken otic vesicle the anlage of anterior and posterior cristae, marked by *Bmp4* expression, arises just next to *SOHo1* expression domain.

1.3.3. Later morphogenesis: from otic vesicle to membranous labyrinth

The sculpting of the otic vesicle into a complex membranous labyrinth occurs in a relatively short time. All the main morphological structures form between stages 20–30 according to Hamburger and Hamilton (HH) (described in Bellairs and Osmond, 1998) in the chicken and during embryonic days (E)10.5–E13.5 in

the mouse. Further development involves mainly cellular differentiation and growth of the labyrinth (Martin and Swanson, 1993; Bissonnette and Fekete, 1996). The key to rapid morphogenesis is hidden in the early segregation of the otic vesicle underlying the further development of distinct inner ear structures. The dorso-lateral domain of the otic vesicle is predetermined to form semicircular ducts, the central part of the medial wall gives rise to vestibular sensory epithelia, and the ventral portion of the otocyst develops into the cochlear duct in mammals and birds (Swanson *et al.*, 1990; Rinkwitz *et al.*, 2001).

1.3.3.1. Endolymphatic duct

The endolymphatic duct is the first structure, which grows out from the spherical otic vesicle. It forms in the dorso-medial region just adjacent to the closure point, and the closure event is absolutely required for the outgrowth (Cecconi *et al.*, 2004).

The signalling from the hindbrain is thought to be essential in endolymphatic duct formation. Namely, the A/P boundary within the endolymphatic duct is aligned with the r5/r6 boundary of the hindbrain and the precise level of Fgf3 secreted from these rhombomeres is thought to be essential for endolymphatic outgrowth. Accordingly, Mansour *et al.* (1993) demonstrated that *Fgf3* knockout mice do not develop an endolymphatic duct. Because of the failure to drain the endolymphatic fluid appropriately, the ear expands into a large cyst. Similarly, the *kreisler* mutant does not express *Fgf3* in the hindbrain, and it lacks the endolymphatic duct outgrowth (McKay *et al.*, 1996).

It is known that several genes expressed in the dorsal otic epithelium are necessary for the development of the endolymphatic duct. For example, hindbrain induced *Gbx2* and its downstream gene *Dlx5* are important in endolymphatic duct formation (Acampora *et al.*, 1999; Lin *et al.*, 2005; Riccomagno *et al*., 2005).

1.3.3.2. Semicircular ducts

The morphological formation of the semicircular ducts starts with the outgrowth of the dorsal otic vesicle epithelium resulting in bilayered epithelial outpocketings by E11.5 in the mouse and by HH25 in the chicken. The formation of tubular semicircular ducts from these outpocketings requires local detachment of their opposite walls and disruption of the underlying basal lamina, allowing the cells to intermingle into a temporary fusion plate structure. In the mouse and the zebrafish the fusion plate cells are thought to disappear via resorption into the duct rim. On the other hand, these cells are removed by apoptosis in the chicken and *Xenopus* (Martin and Swanson, 1993; Fekete, 2004). As a result, hollow ducts are created.

The study by Cecconi *et al*. (2004) demonstrated that apoptosis is essential for normal cellular proliferation required for the outgrowth, and apoptosis-deficient *Apaf1* mouse mutants develop semicircular ducts reduced in size. This surprising result suggested that apoptosis might stimulate proliferation through increasing locally the amount of growth factors (Cecconi *et al.*, 2004).

Also, the role of Fgf10 signalling has been shown to play an important role in semicircular duct outgrowth. Using fate-mapping studies in chicken otic vesicle, Chang *et al*. (2004) identified genesis zones of the semicircular ducts adjacent to prospective cristae, which contribute to the outgrowth of the ducts. They showed that the expression of *Fgf10* in chicken presumptive cristae is responsible for the formation of this zone by inducing or upregulating *Bmp2* expression. Accordingly, mice with targeted mutation in *Fgf10* locus exhibit severe agenesis of the semicircular ducts (Pauley *et al*., 2003).

Importantly, a laminin-related axon guidance molecule, Netrin1, has been shown to be essential for fusion plate formation. There is strong evidence that this secreted protein is simultaneously required for the detachment of the fusion plate epithelium from the basement membrane as well as for the stimulation of the proliferation of adjacent mesenchymal cells needed to push the forming fusion plate epithelial walls together (Salminen *et al.*, 2000). In the same way, Fgf9 produced in fusion plate epithelia, is thought to be involved in the fusion event through its mesenchymally expressed receptors Fgfr1(IIIc) and Fgfr2(IIIc), which have an effect on mesenchymal proliferation (Pirvola *et al.,* 2004). The fact that the expression of *Netrin1* and *Fgf9* are independent of each other (Pirvola *et al.,* 2004; Matilainen and Salminen unpublished data) indicates that at least two parallel pathways are involved in promoting the mesenchymal proliferation during the development of the semicircular duct.

Hmx2 and *Hmx3* are expressed in the dorso-lateral portion of the closed otic vesicle and inactivation of either of these genes results in the loss of the semicircular ducts. Nor does the fusion event occur in these mutants, but the phenotype is somewhat different than in the *Ntn1* mutant. The *Hmx2* and *Hmx3* mutants express *Netrin1,* and thus the fusion plate epithelia are able to detach from the underlying basement membrane and come close to each other, whereas the fusion event fails (Hadrys *et al.*, 1998; Salminen *et al.*, 2000; Wang *et al.*, 2001).

In addition to Netrin1 and Fgf9, Prx1 and Prx2 are also involved in mesenchymal-epithelial interaction during the development of the semicircular duct. The strong expression of these genes colocalizes in the lateral periotic mesenchyme, and the ablation of both *Prx* genes causes the loss of the lateral duct and exhibits thickened vertical ducts (ten Berge *et al.*, 1998).

Mutations affecting semicircular duct formation often cause changes of different character in particular ducts, indicating that their morphogenesis is directed via segregated molecular mechanisms. For example, *Dlx5* is expressed dorsally in the otic vesicle, and its absence results in the total loss of the two vertical ducts, whereas the lateral duct develops normally (Acampora *et al.*,

1999). On the other hand, Otx1 is the factor that is needed specifically for the development of the lateral semicircular duct. It is expressed in a rather small postero-ventro-lateral domain of the otic vesicle and is absent from lampreys, which do lack the lateral canal system. Also zebrafish with the mutated *Otx1* gene exhibit an ear very similar to lampreys (Hammond and Whitfield, 2006).

1.3.3.3. Cochlea

The cochlear duct together with the saccule starts to grow as a hollow tube out of the medio-ventral aspect of the otic vesicle at E11.5 in the mouse and at HH23 in the chicken (Martin and Swanson, 1993; Bissonnette and Fekete, 1996). A specific domain undergoing apoptosis is found in the ventro-medial wall of the otic vesicle in mammals and birds. Such focal cell death has not been found in other vertebrates (*Xenopus*, zebrafish), suggesting a possible association with cochlear outgrowth (Bever and Fekete, 1999; Cecconi *et al.*, 2004; reviewed in Leon *et al*., 2004). Indeed, using mice with ablated *Apaf1*, Cecconi *et al.* (2004) demonstrated that the lack of apoptosis in these animals causes a severe reduction in the cochlear duct. As in the vestibule, cell proliferation was reduced in the outgrowing cochlear duct in apoptosis deficient otic epithelium.

Later the cochlea becomes separated form the saccule by the cochleo-saccular duct. In the mouse the formation of this constriction requires programmed cell death (Cecconi *et al.*, 2004).

Several transcription factors are involved in cochlear duct morphogenesis. *Pax2* is expressed in a broad medio-ventral portion of the otic vesicle. Later it is expressed in mouse cochlea as well as in the saccule, utricle and the endolymphatic duct. Interestingly, the cochlear duct is selectively sensitive to the lack of this factor (Favor *et al.*, 1996; Torres *et al.*, 1996; Fekete, 2004).

Eya1 is expressed throughout the early otic region and becomes restricted to the ventro-medial wall of the vesicle. Mice carrying a hypomorphic allele of *Eya1* (*Eya*^{bor}) develop a truncated cochlear duct whereas the dorsal part remains rather intact (Johnson *et al.*, 1999). Likewise, branchio-oto-renal (BOR) and BO syndromes in humans are caused by allelic defects of *EYA1*, exhibiting a similar cochlear defect (Abdelhak *et al.*, 1997).

Little is known about the molecular mechanisms responsible for cochlear coiling in mammals and regulation of the variation in the number of coils between different species. However, some targeted mutants exhibit an abnormal cochlear shape. Otx1 and Otx2 are expressed ventro-medially in the early otic vesicle and are involved later in the correct coiling of the cochlear duct in a dose-dependent manner (Morsli *et al.*, 1999). *Pou3f4* mediates cochlear duct coiling indirectly influencing the differentiation of the adjacent mesenchyme (Phippard *et al.*, 1999; reviewed in Kiernan *et al.*, 2002).

1.3.4. Sensorineural determination and differentiation

Inner ear hair cells are secondary sensory cells as they are innervated by the primary sensory neurons located in the cochleo-vestibular ganglion. The sensory cells and neurons originate from a common antero-ventro-medial quadrate of the early otic epithelium (reviewed in Fekete and Wu, 2002). One of the earliest genes marking the sensory and neural competent region in mouse and chicken otic epithelium is *Fgf10*, followed by several members of the Notch signalling pathway (Pirvola *et al.*, 2000; reviewed in Fekete and Wu, 2002; Alsina *et al.*, 2004).

In the context of evolution, Fritzsch and Beisel (2004) proposed that both the vertebrate hair cells and their innervating neurons have been derived from ciliated mechanosensory cells like those found in insect scolopidal organs. If it were the case, vertebrate hair cells and neurons should originate from the same progenitors. A recent clonal analysis in chicken otic epithelium has demonstrated that sensory hair cells and neurons can originate from common progenitors. However, origin from separate lineages is more common (Satoh and Fekete, 2005).

1.3.4.1. Development of the cochleo-vestibular ganglion

The precursors of sensory neurons delaminate from the otic epithelium, emigrate, and divide as neuroblasts, and coalesce to form a fused cochleo-vestibular ganglion (reviewed in Torres and Giraldez, 1998). Clonal analysis has shown that the auditory and vestibular neuroblasts may arise from the same precursors (Satoh and Fekete, 2005). However, it is not known when exactly the auditory and vestibular neuronal fates are distinguished. Later the fused ganglion separates into vestibular and auditory parts (reviewed in Torres and Giraldez, 1998).

After becoming postmitotic each neuron makes a synaptic connection with one of the sensory organs and with appropriate brainstem neurons of either auditory or vestibular nuclei (Lang and Fekete, 2001; reviewed in Fekete and Wu, 2002; Rubel and Fritzsch, 2002). The mechanisms guiding neuroblast migration and later its axon pathfinding back to the otic epithelium are largely unknown (Satoh and Fekete, 2005).

Vertebrate transcription factors of the basic Helix-Loop-Helix (bHLH) family are essential in initiating the neuronal lineages, promoting neuronal precursor delamination from the ectoderm that has been already specified towards the neural fate, and in neuronal differentiation (reviewed in Bertrand *et al.,* 2002). In the otic vesicle epithelium a domain expressing *Fgf10* is specified as the sensorineural competent region in mice and chickens (Pirvola *et al*., 2000; Alsina *et al*., 2002). In the chicken, Fgf10 has been shown to promote neuronal fate by regulating positively the expression of two *bHLH* genes,

Neurogenin 1 (Ngn1) and *NeuroD* (Alsina *et al.,* 2002). In mouse otic epithelium, Ngn1 is absolutely essential in neuronal specification, and *Ngn1–/–* mouse embryos do not develop any neuroblasts (Ma *et al.,* 2000). Recent work by Matei *et al.* (2005) proposes that the role of Ngn1 is to switch the sensorineural precursors towards the neural fate (see also 1.3.4.2.). On the other hand, NeuroD is not required for establishing the neural cell fate but is involved in neuron precursor delamination instead. Accordingly, in *NeuroD* mutants the neuroblasts form but fail to emigrate from the otic epithelium (Liu *et al.,* 2000; Kim *et al.,* 2001).

The expression of proneural genes in neural progenitor cells is known to prevent neighbouring cells from taking the same fate. This process, named lateral inhibition, is achieved through activating the Notch signalling pathway. In the developing nervous system the activity of proneural genes results in upregulating Notch ligand expression in future neural progenitors that subsequently activate Notch signalling in adjacent cells. As a result, activated Notch signalling leads to upregulating the expression of proneural gene repressors, such as members of the *Hes* gene family, and thereby inhibiting the cell from differentiating into a neuron (reviewed in Bertrand *et al.,* 2002).

Notch-signalling plays a major role in judging between the neural versus otic epithelial fate in inner ear development. In particular, *Notch1* is widely expressed within the otic placode and vesicle whereas one of its ligands, Delta1, is present in future neuroblasts. *Delta1*-expressing neuroblasts inhibit their neighbours from taking the same fate and therefore control the number of neurons (reviewed in Fekete, 2004). In agreement with this interpretation, there is an excessive number of ganglionic neurons in zebrafish *mind bomb* mutant where Delta-Notch signalling is inhibited (Haddon *et al.,* 1998).

Survival of the neurons of auditory and vestibular ganglia depends on neurotrophins secreted from the sensory epithelia that they innervate; the neurons associated with cristae require brain-derived neurotrophic factor (BDNF), whereas cochlear neurons depend on BDNF and neurotrophin 3 (NT3) (Fritzsch *et al*., 1997; 1999).

1.3.4.2. Sensory determination

In addition to the failure in neuron formation, *Ngn1* mutants exhibit severe reduction in hair cell production (Ma *et al.*, 2000). Recent work by Matei *et al.* (2005) proposes a role for Ngn1 in choosing between neuronal and hair cell fates. The authors suggest that *Ngn1* is expressed in common sensorineural precursors and switches the neural fate in while its absence skews cell fate decision towards hair cells. The reduction of sensory epithelia in *Ngn1* knockout mice is caused by an earlier terminal mitosis of hair cell precursors. Thus, the early development of sensory neurons and sensory epithelia cannot be considered independently.

Notch-signalling pathway members are among the first genes expressed in future sensory anlage in the mouse, chicken, and the zebrafish. The expression of a modulator of the Notch pathway, *Lfng* marks the sensory competent ventro-medial part in the chicken and mouse (Morsli *et al.*, 1998; Cole *et al.*, 2000). Likewise, the gene encoding Notch ligand Jagged1, *Jag1*, is expressed throughout the prosensory region (Adam *et al.*, 1998; Morrisson *et al.*, 1999). Recently Brooker and colleagues (2006) demonstrated that the loss of Jag1 leads to severe reduction of sensory epithelium, suggesting that the activation of Notch signalling by Jag1 in early otic epithelium induces prosensory fate.

Besides Notch-signalling, a member of the B Sox family, Sox2 has been recently shown to be involved in prosensory domain specification. Two mouse mutants with missing or reduced *Sox2* expression, *Lcc* and *Ysb*, respectively, either fail to establish the prosensory domain and their hair and supporting cells do not develop at all *(Lcc/Lcc)* or they develop in reduced numbers and disturbed arrangement *(Ysb/Ysb)* (Kiernan *et al.*, 2005a).

1.3.4.3. Hair cell development

Clonal analysis in the chicken has demonstrated that hair and supporting cells inside the sensory epithelium arise from the same precursors (Fekete *et al.*, 1998). Notch-signalling is thought to determine also the hair versus supporting cell fate. Unlike the potential inductive role of Notch-signalling in prosensory fate determination (Brooker *et al*., 2006), its further role inside the prosensory epithelium is inhibition. The proposed lateral inhibition model suggests that cells within the sensory organ anlage are equipotential about their fate expressing *Notch1* and, to lesser extent, its ligand(s). For unknown or stochastic reasons some of these cells upregulate ligand expression and activate Notch signalling in the surrounding cells. The activation of Notch leads to hair cell fate inhibition and the cells differentiate into supporting cells (Kiernan *et al*., 2002). The expression of the genes encoding Notch ligands Jagged2 (Jag2) and Delta1 in hair cells and *Notch1* in supporting cells is consistent with this model (Lanford *et al.,* 1999; Morrison *et al.,* 1999). Moreover, *Jag2* knockout mice produce extra rows of hair cells in the cochlea indicating the failure of lateral inhibition (Lanford *et al.*, 1999), and the loss of Delta1 leads to premature and excessive hair cell production in the cochlea (Brooker *et al.*, 2006). Even more extreme is the phenotype in $Jag2^{-/-}/Delta1^{hyp/-}$ mutant cochleae that exhibit supernumerary and extremely disorganized hair cells (Kiernan *et al.*, 2005b). The zebrafish *mind bomb* mutant strongly supports this hypothesis as inhibition of Notch signalling leads exclusively to hair cell differentiation inside the sensory patches (Haddon *et al.*, 1999). Nevertheless, the situation cannot be approached in a too simplified manner. For example, Notch ligand *Jag1* is selectively expressed in supporting cells, and *Delta1* mutant mice develop both

supporting cells and hair cells in excess (Morrison *et al.*, 1999; Brooker *et al.*, 2006).

Further differentiation of hair cells depends mainly on bHLH transcripton factor Math1 (homologue to *Drosophila* atonal1). *Math1* knockout mice lack completely the sensory hair cells (Bermingham *et al.*, 1999). Moreover, over-expression of Math1 induces hair cell generation in neonatal rat cochlear explants as well as in guinea pig inner ears *in vivo* (Zheng and Gao, 2000; Kawamoto *et al*., 2003). On the other hand, the *Hes* bHLH genes, being downstream targets of Notch-signalling, inhibit hair cell generation via negative regulation of *Math1* (reviewed in Kelley, 2006). Accordingly, knockouts for *Hes1* and *Hes5* develop supernumerary hair cells in the cochlea (Zine *et al*., 2001). Terminal differentiation of hair cells is dependent on the expression of a Pou domain factor gene *Brn3.1* that follows *Math1* (Erkman *et al.*, 1996; Xiang *et al*., 1998).

1.3.4.4. Specification and development of distinct sensory organs

The specification of a particular sensory organ within a prosensory primordium has remained largely unknown. According to the expression pattern of some known early sensory markers, which are confined only to a particular set of the sensory primordia, it is possible to deduce the approximate timing of the specification of a particular sensory organ. For example, *Bmp4* is selectively confined to the primordia of the cristae at E10.5, and the *Lfng* expression domain is specifically confined to the primordia of the maculae and the organ of Corti in mouse otic epithelium by E12 (Morsli *et al.*, 1998) indicating that these sensory epithelia have been specified by these stages.

As noted, signalling across the boundaries of different gene expression domains may play a role in determining the identity and positions of distinct sensory epithelia. However, there is no molecular proof of this hypothesis.

Interestingly, activating the canonical Wnt-signalling pathway in chicken otic vesicle prior to sensory organ specification caused the formation of ectopic patches of hair cells in non-sensory regions and additionally converted the auditory patches into the vestibular ones (Stevens *et al*., 2003). However, it is not known whether the endogenous Wnt pathway is involved in sensory organ specification. Instead, Wnt members have been shown to play a role in determining hair cell polarity (Dabdoub *et al.,* 2003; Dabdoub and Kelley, 2005).

The mammalian auditory sensory epithelium (the organ of Corti) is much more complex than the other sensory epithelia. Two types of hair cells (inner and outer hair cells) are arranged in rows and are surrounded by two types of highly specialized supporting cells, the pillar and Deiter's cells (reviewed in Barald and Kelley, 2004; Figure 3D). The molecular mechanisms involved in the cytodifferentiation of the organ of Corti have remained largely unknown. Several members of the Fgf-signalling pathway are expressed in developing

auditory sensory epithelium, indicating their role in its differentiation. For example, Fgfr3 has been shown to be involved specifically in pillar cell specification; these cells separate IHC and OHC in the organ of Corti (Colvin *et al*., 1996). In contrast Fgfr1 is thought to promote specifically OHC development (Pirvola *et al.,* 2002).

1.4. Gata transcription factors in development

The precise combined application of transcription factors is a key to achieving a high spectrum of cell types and tissues from multipotent cells during development. The Gata transcription factor family, although containing rather few members with similar DNA binding properties, plays numerous vital and unique roles during development. Remarkably, Gata factors have been shown to be involved in cell-fate specification, cell proliferation, differentiation, as well as cellular movements (reviewed in Patient and McGhee, 2002).

Members of the Gata family have been described across eukaryotes: six Gata factors (Gata1–6) have been characterized in vertebrates, four in *Drosophila*, and eleven in *C. elegans*. The name Gata originates from the common feature of its members to bind the consensus DNA sequence (A/T) GATA (A/G) (reviewed in Patient and McGhee, 2002; Cantor and Orkin, 2005).

Gata proteins contain either one or two highly conserved zinc finger (ZF) domains. While the C-terminal ZF is responsible for binding to GATA sites in its target genes, the N-terminal ZF can modulate the binding specifity via interacting with transcriptional coregulators. Notably, Gata N-ZF interacts both in vertebrates and flies with transcriptional coregulators belonging to the Friend of Gata (FOG)/U-shaped (Ush) family. This interaction can either repress or enhance Gata-mediated transactivation depending on the cell and promoter context. Gata factors can also remodel chromatin structure further away from the promoters and modulate their transcriptional competence (reviewed in Patient and McGhee, 2002; Grass *et al.,* 2003; Cantor and Orkin, 2005; Shoemaker *et al.,* 2006).

The six vertebrate Gata factors can be divided into two subgroups regarding their amino acid sequence and major roles in certain tissues. Gata-1/2/3 possess important roles in haematopoiesis while Gata-4/5/6 are involved in heart and gut development. Additionally, these factors are involved in numerous other tissues (reviewed in Patient and McGhee, 2002; Cantor and Orkin, 2005; Burch, 2005).

Regarding the particular interest of the present thesis, the roles and molecular pathways concerning Gata2 and Gata3 will be discussed below.

1.4.1. Gata2 and Gata3 in development

Gata2 and *Gata3* are expressed in a variety of tissues during embryogenesis in a highly restricted spatio-temporal manner. Dissecting their roles, however, has been complicated due to the early embryonic lethality of their knockouts. Particularly, the loss of Gata2 in mice leads to death around E10.5 due to severe anaemia (Tsai *et al.,* 1994), whereas *Gata3* mutants die about at the same stage (E10.5–11.5) with defects in several organ systems (Pandolfi *et al.,* 1995). The primary cause leading to early lethality of *Gata3–/–* embryos is the noradrenaline deficiency of the sympathetic nervous system leading to early cardiac failure (Lim *et al.,* 2000).

The best-characterized roles and molecular pathways involving Gata2 and Gata3 together with Gata1 concern blood development and exemplify how the transcription factors with similar properties play unique roles. Gata2 is essential for the proliferation and maintenance of haematopoietic progenitor cells (Tsai and Orkin, 1997). Additional differentiation of the progenitor cells requires the downregulation of *Gata2* (Persons *et al.,* 1999). In particular, the expression of *Gata2* is induced in response to Bmp4 signalling in haematopoietic progenitors where it maintains its own expression. The positive autoregulation does not involve the binding of Gata2 to either of its two promoters; instead, a distant upstream region is required. Gata2 is likely to induce the expression of *Gata1*, the latter being specifically required for *Gata2* repression and subsequent definitive haematopoiesis. In fact, Gata1 interacting with FOG1 displaces rapidly Gata2 from the *Gata2* upstream region, which disrupts the positive autoregulation and establishes a repressive chromatin structure (Im *et al.*, 2005; Grass *et al.*, 2003 and references therein).

In contrast to Gata2, which is required for the development of haematopoietic precursor cells, Gata3 is involved in T helper (Th) cell fate specification. Gata3 promotes Th2 cell fate by activating the expression of Th2 specific cytokines (IL-5, IL-4, IL-13, IL-10) and inhibits the expression of IFN-γ that is required for Th1 specification (Zheng and Flavell, 1997; Ferber *et al.,* 1999; Blokzijl *et al.,* 2002; Shoemaker *et al.,* 2006).

Gata2 and *Gata3* are the only family members expressed in the developing brain. Both genes are expressed in many types of neural precursors as well as in postmitotic neurons, and their roles in the specification of distinct neural populations have been demonstrated (Nardelli *et al*., 1999; Pata *et al*., 1999; Zhou *et al*., 2000; Karunaratne *et al*., 2002; Craven *et al*., 2004; Tsarovina *et al*., 2004). Pata *et al*. (1999) showed that in ventral r4 the expression of *Gata3* is dependent on Gata2, the latter being under the positive control of Hoxb1. This molecular cascade is responsible for the migration of facial branchiomotor neurons to r6 as well as the projection of vestibuloacoustic efferent neurons to the ear, both of which develop in r4. The study of El Wakil *et al*. (2006) shows that unlike its role in the haematopoietic precursor cells, Gata2 inhibits the proliferation of neural progenitors in the embryonic spinal cord.

As in haematopoietic and nervous systems, Gata3 has been shown to be involved in cell lineage determination in skin multipotent progenitor cells (Kaufman *et al.*, 2003). Gata3 plays an essential role also in kidney development. Particularly, Gata3 is required for the morphogenesis and guidance of the Wolffian duct, and the homozygous mutants fail to form the metanefros (Grote *et al.,* 2006). A vital but different role in urogenital development has been demonstrated for Gata2. *Gata2–/–* mutants complemented with a YAC comprising a partial genomic *Gata2* fragment can overcome lethality and exhibit deformed kidneys and urethras leading to a blockade in urine excretion (Zhou *et al.*, 1998).

1.4.2. Gata3 in the inner ear

Gata3 is expressed in a highly localized manner during the inner ear development both in the otic epithelium and the surrounding mesenchyme and it performs a number of different roles (Karis *et al.,* 2001; Lawoko-Kerali *et al.*, 2002; 2004; van der Wees *et al.,* 2004).

The development of Gata3 deficient mouse ears arrests in otic vesicle stage; the phenotype is consistent with strong *Gata3* expression in early otic tissue. Remarkable variability has been detected in this early otic phenotype, as well as in embryo survival (Karis *et al.,* 2001).

Reduction of primary otic neurons has also been recorded in *Gata3* mutants. This feature might be linked with general reduction of otic epithelium or, most likely, with selective loss of auditory neurons. In particular, *Gata3* is specifically expressed in auditory ganglionic neurons in normal animals where it may distinguish auditory neuron progenitors from the vestibular ones as early as in otic epithelium, and it is required for NeuroD expression/maintenance during auditory neuroblast migration (Karis *et al.,* 2001; Lawoko-Kerali *et al.,* 2004).

Additionally, Gata3 has a role in axon navigation from r4 neuron population, which provides an efferent innervation to otic sensory epithelia (Karis *et al.,* 2001).

Gata3 possesses also an important role in hair cell maintenance. Mutations in the human *GATA3* gene result in the HDR syndrome, which exhibits sensorineural deafness in addition to hypoparathyroidism and renal dysplasia (van Esch *et al.,* 2000; Lichtner *et al*., 2000). Similarly, mice carrying only one intact *Gata3* allele suffer from progressive hearing loss caused by poor maintenance of cochlear hair cells (van der Wees *et al.,* 2004).

2. AIMS OF THE STUDY

As noted, certain knowledge has been gathered about the roles of Gata3 in ear morphogenesis, sensory neuron development, and hair cell maintenance. However, the molecular bases underlying these processes have remained largely unknown, and the target genes controlled by Gata3 in the inner ear have not been described as yet. In addition, the expression and the role of the closely related factor *Gata2* possessing similar DNA binding properties has not been described, and the potential regulation and redundancy between these factors in ear development has remained elusive.

In the context of evolution an important question arises concerning the molecular conservation leading to the formation of the membranous labyrinth in mammals and birds, whose common ancestor dates back to around 350 million years. While many important genes for the inner ear development of the mouse have been identified using gene-targeting technologies, little is known about the molecular pathways responsible for otic development in birds and even less about the functional conservation of regulatory pathways between the two vertebrate classes. As the mammalian cochlea is unable to regenerate (reviewed in Fritzsch *et al*., 2006b), the capability of the avian auditory organ to replace damaged hair cells (Stone and Rubel, 2000) is of great interest. Comparative information of genes expressed in otic sensory epithelia in different animal classes might provide important knowledge when designing new therapies for human deafness.

Additionally, chickens are easy to manipulate *in vivo* during embryogenesis, making this species an attractive model for studying organ development. For example, several studies on the cellular movements underlying ear formation have been performed in birds. In order to extrapolate the gained knowledge to mammals, comparative expression pattern analyses are required.

The present study has the following aims:

1. To describe the formation and patterning of *Gata3–/–* mouse otic vesicle;
2. To identify target genes for Gata3 in mouse ear development;
3. To study the role of Gata3 in vestibular and auditory sensory differentiation in the mouse;
4. To analyse the *Gata2–/–* mouse otic phenotype;
5. To compare the spatio-temporal inner ear expression patterns of murine *Gata3* and *Gata2* with their chicken counterparts.

3. RESULTS AND DISCUSSION

3.1. *Gata3* is essential for normal otic morphology during invagination (I, II, and unpublished data)

Gata3–/– mouse embryos are known to form aberrant otic vesicles that are unable to develop any advanced structures (Karis *et al.,* 2001). Whether the impaired otic development is a consequence of abnormal cell proliferation and/or survival, intraepithelial rearrangement or differentiation, has remained unknown. In order to obtain more insights into the nature of the failure in inner ear morphogenesis, the first morphogenetic steps of *Gata3–/–* inner ears when the placode forms an otic vesicle were studied. In the used *Gata3* mutant mice, the *nlsLacZ* sequence has been inserted into the *Gata3* locus, and the activity of β-galactosidase does reflect the endogenous *Gata3* expression (Hendriks *et al*., 1999; Karis *et al*., 2001). Since *Gata3+/–* inner ears develop morphologically normally, they were used as reference. Also, the activity of β-galactosidase during the invagination process in *Gata3+/–* and *Gata3–/–* mutant embryos was followed.

The onset of *Gata3* expression in the otic region appeared in the morphologically distinguishable otic placode in 12-somite stage embryos E8.5 (II). It was earlier than the expression in the adjacent hindbrain starting at E9.0 (Nardelli *et al.*, 1999; Pata *et al.*, 1999) and in the periotic mesenchyme at E10.5 (I). The absence of *Gata3* expression in the preplacodal otic region and in the adjacent tissues indicates that the formation of the otic placode occurs without Gata3.

Small morphological alterations were detectable in approximately half of the *Gata3–/–* otic placodes already around E8.5, at the onset of *Gata3* expression in wild type (wt) embryos (II). The thickness of the *Gata3–/–* otic placodes was not uniform as in wt and *Gata3+/–* mouse embryos; the dorsal part was thinner than the ventral one (II; Figure 4). At E8.75–E9.0, the alterations in size and shape of the otic cups were obvious in all *Gata3–/–* embryos (II; unpublished data).

The closing *Gata3–/–* otic cups contained on average 17 per cent less cells per section compared to wt cups. The shape of the *Gata3–/–* otic cups was variable and differed from wt and *Gata3+/–* littermates. Namely, while the wt and *Gata3+/–* cups were spherical, the mutant cups were either dorso-ventrally narrowed, or they presented an ectopic morphological boundary in the medial wall (II; Figure 4). This boundary divided the cup area into the dorsal and the ventral part, and the invagination process of the dorsal domain often lagged behind in comparison with that of the ventral domain.

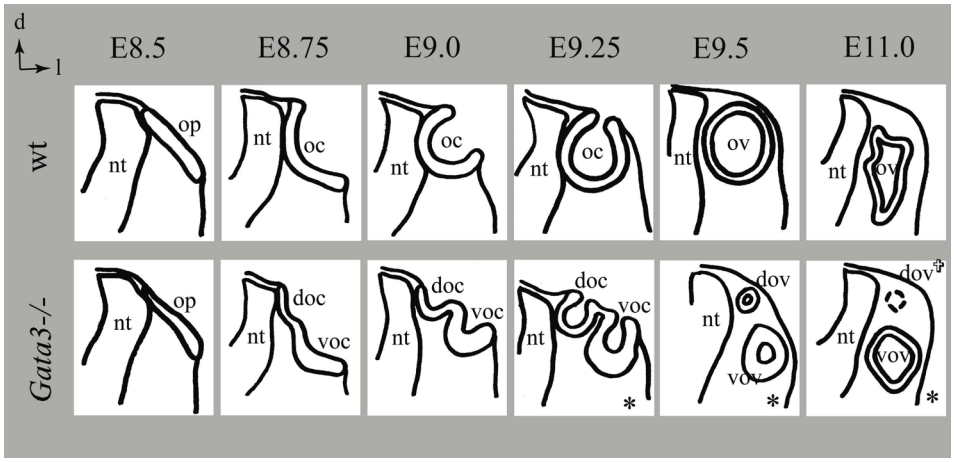

Figure 4. Invagination of wt and *Gata3–/–* otic placodes. The otic placode arises next to the neural tube; it invaginates to form the otic cup that closes into the otic vesicle, which subsequently undergoes a rapid morphogenesis. During the invagination the medial domain of the otic epithelium exhibits an attachment to the neural tube. The morphology of the otic placode and cup is different in *Gata3–/–* embryos, and an ectopic boundary often occurs along the ventro-medial wall of the closing otic epithelium. The invaginating otic epithelium exhibits reduced attachment to the neural tube, and the ventro-medial domain remains more distant from the neural tube than wt littermates. During otic closure the epithelium disrupts along the ectopic morphological boundary, and as a result two separate vesicles form. The dorsal vesicle disappears via apoptosis. Schematic sections, orientation shown in left upper corner, not in scale. The genotypes and embryonic stages are indicated. Abbr: d, dorsal; doc, dorsal otic cup; dov, dorsal otic vesicle; dov†, dorsal otic vesicle disappears via apoptosis; l, lateral; nt, neural tube; oc, otic cup; op, otic placode; ov, otic vesicle; voc, ventral otic cup; vov, ventral otic vesicle; * the dorsal and ventral otic vesicles are schematically shown on the same level of the section.

In general, little is known about the mechanisms influencing the process of otic placode invagination. Attachment of the otic cup epithelium to the neural tube basal lamina is thought to be necessary for normal invagination in the chicken (Brown *et al*., 1998; Moro-Balbás *et al*., 2000; Visconti and Hilfer, 2002). A close connection was detected between the ventro-medial part of the otic cup epithelium with the strongest *Gata3* expression and the neural tube at E9.0 in wt and *Gata3+/–* mouse embryos. In contrast, in *Gata3–/–* embryos the ventro-medial part of the otic cup remained distant from the neural tube. The dorso-medial otic cup epithelium exhibited attachment to the neural tube both in wt and *Gata3–/–* embryos, but the contact area was remarkably reduced in the mutants (Figure 4).

In spite of the abnormal morphology of the otic cup, the otic epithelium was able to close in *Gata3* mutants. The resulting vesicles were smaller and often elongated in shape compared to the wt or *Gata3+/–* ones. Moreover, in about

half of the *Gata3* mutants two separate vesicles formed on one side of the head (either on one side or both). In these embryos, disruption of the epithelium occurred along the observed ectopic morphological boundary. When two vesicles were present, the dorsal one was always smaller than the ventral one, and the relative position of the two vesicles was constant (II; Figure 4). At E11.5 no separate dorsal vesicle could be detected in *Gata3* mutants (II).

In conclusion, Gata3 is required for early otic epithelium morphogenesis, and since *Gata3* was not expressed in adjacent tissues during otic vesicle formation, there is strong likelihood that its effect is placode-autonomous.

3.2. Cell proliferation is altered, but apoptosis occurs normally in *Gata3* deficient otic epithelium (II)

Normal morphogenesis and growth of the inner ear requires precise control of programmed cell death and proliferation (Cecconi *et al*., 2004). As the Gata3 deficient otic cups and resulting vesicles were always smaller than those of wt and *Gata3*+/– littermates, cell proliferation and death were compared between wt and *Gata3*–/– otic epithelia.

A high and uniform proliferation rate was detectable in wt otic cup and early otic vesicle epithelia (E9.0–E9.5). At E10.5 one could observe changes in the proliferation pattern. While the main part of the vesicle epithelium proliferated highly, only little proliferation occurred in the dorsal, outgrowing endolymphatic duct domain (II).

A small (10–15%) reduction in the cell proliferation rate was observable in Gata3 deficient otic epithelium at E9.0–9.5. Moreover, the dorsal part of the *Gata3*–/– cups with the ectopic morphological boundary always had fewer proliferating cells than the ventral part. This situation resembled what can be seen in E10.5 wt embryos where the endolymphatic duct domain proliferated less than the remaining vesicle (II). The different proliferation rates in the dorsal and ventral domains of the *Gata3*–/– otic cup could imply that the endolymphatic duct differentiation could occur precociously in Gata3 mutants. However, the additional findings of this study show that it is not the case. Rather, the reduced contribution of highly proliferating ventral cells to the dorsal compartment accounts for the precocious endolymphatic characteristics. At E10.5 the separated dorsal vesicle in *Gata3* mutants had a low proliferation rate similar to the endolymphatic duct domain in wt littermates (II). The ventral vesicle exhibited approximately 20% less proliferating cells than the corresponding domain of the wt vesicles (II), suggesting that the separate dorsal vesicle might comprise mainly of the endolymphatic duct domain.

In conclusion, the data show that Gata3 is required for normal proliferation in the main part of the otic vesicle, but not in the dorsal, endolymphatic domain. Gata3 could regulate the proliferation either directly since it is expressed all

through the early otic epithelium at the stage when the proliferation differences appear between wt and *Gata3* mutants, or via changes in epithelial properties, such as adhesion, which is altered in Gata3 deficient otic epithelium (see 3.5).

Apoptosis occurs in restricted areas of the developing otic epithelium in the mouse. At the otic vesicle stage, apoptosis is detected mainly in two areas including the dorsally outgrowing endolymphatic duct and a dorso-lateral domain (Cecconi *et al*., 2004). Unlike cell proliferation, one could not detect any significant differences in the proportion and the distribution of apoptotic cells between the wt and *Gata3–/–* otic epithelia at E10.5. In case two vesicles occurred on one side of the head in *Gata3–/–* embryos, the dorsal vesicle contained a high proportion of dying cells corresponding to the proportion observed in the wt endolymphatic duct domain (II).

Fate-mapping studies have shown that the endolymphatic duct outgrowth is largely accomplished by migration of the cells from the ventral part of the otic epithelium (Brigande *et al*., 2000a). In case the otic epithelium has disrupted into two parts, the ventral cells cannot contribute to the presumptive endolymphatic domain, and subsequently the separated dorsal vesicle (the endolymphatic domain) disappears via apoptotic cell death (Figure 4).

3.3. Gata3 is not required for general patterning of the early otic epithelium (I, II, and unpublished data)

The early failure in *Gata3–/–* otic development could be a result of patterning defects, leading to the failed predetermination of inner ear structures in otic epithelium. In order to understand to what extent Gata3 is necessary for the patterning of the otic epithelium and to what extent the patterning is preserved in the epithelium that has disrupted into two vesicles, the expression domains of the genes belonging to the evolutionarily conserved *Pax-Eya-Six-Dach* regulatory network (reviewed in Wawersik and Maas, 2000) were verified. The members of this hierarchical network are broadly expressed during inner ear development across the vertebrate classes, exhibiting asymmetrical expression domains in the otic vesicle (Ozaki *et al.*, 2003; Zou *et al.*, 2006).

The *Pax-Eya-Six-Dach* regulatory hierarchy is partially conserved during mouse inner ear development. Two members of the paired box (Pax) transcription factor family, *Pax8* and *Pax2,* are the earliest genes activated specifically in mouse otic ectoderm followed by a homologue to *Drosophila eyes absent* gene, *Eya1*. However, it is not known whether the *Eya1* expression is dependent on the Pax factors. Eya1 is demanded for initiating the expression of a homologue to *Drosophila sine oculis*, *Six1*. The expression of *Drosophila dachshund* homologues *Dach1* and *Dach2* seems to occur independently. Similarly to *Gata3–/–* embryos, mutated *Eya1*and *Six1* genes cause a blockade of ear development at the otic vesicle stage. The severe otic phenotypes in *Eya1*

and *Six1* mutant mice are accompanied by patterning defects (Ozaki *et al.*, 2003; Zou *et al.*, 2006).

A similar *Pax8* expression in wt and in *Gata3–/–* otic placodes at E8.5 could be observed (unpublished data; Figure 5A,B). This was in line with a previous suggestion that Gata3 is not involved in otic placode induction and specification (II). At E9.5, *Pax8* was strongly expressed at the dorso-medial pole of the wt otic vesicles, but the expression was absent from the Gata3 deficient otic epithelium (unpublished data; Figure 5C,D). In wt littermates the down-regulation of *Pax8* happened a day later, at E10.5 (unpublished data). In contrast to *Pax8*, the expression of *Pax2* was not changed in *Gata3–/–* otic vesicles (I). Since the expression of *Pax2* is confined to the medio-ventral domain of the otic vesicle, partly overlapping with the *Pax8* expression domain (Zou *et al.*, 2006), it is unlikely that the complete loss of *Pax8* in Gata3 deficient otic epithelium was caused by alterations in epithelial identity. The earlier downregulation of *Pax8* expression in *Gata3–/–* otic vesicles could indicate the premature epithelial differentiation. However, there is no sufficient data to support this possibility.

In mouse otic vesicle the expressions of *Eya1* and *Six1* are restricted to the ventralmost domain (Ozaki *et al.*, 2003; Zou *et al.*, 2006), and the expression of *Dach1* is initiated in the dorsal domain (Heanue *et al.*, 2002; Ozaki *et al.*, 2003). The expression analyses of this study showed that similarly to *Pax2*, the expression patterns of *Eya1*, *Six1* and *Dach1* were unchanged in *Gata3–/–* otic vesicles (I; II). When two vesicles were present on one side of the head, the expression signals of ventral *Six1* and dorsal *Dach1* were detected in the ventral and dorsal vesicles, respectively (II).

Taken together, the findings of this study suggest that the D/V polarity is preserved in the *Gata3–/–* otic epithelium. The M/L boundary forms in the vesicles that remain intact. However, if the otic epithelium disrupts into two parts, the M/L boundary cannot form normally. Additionally the results of this study suggest that Gata3 functions independently from the *Pax-Eya-Six-Dach* regulatory network in the inner ear.

3.4. Loss of Gata3 leads to reduced expression of dorsal otic genes (II)

The aberrant early development of dorsal otic epithelium made it interesting to study further the endolymphatic characteristics in *Gata3–/–* mutants. In this study wt and *Gata3* mutant embryos were compared for the expression of several dorsal otic genes, which are expressed in the developing endolymphatic duct.

Gbx2 and *Dlx5* are strongly expressed in the dorsal domain of the early otic epithelium of the mouse and later continuously in the endolymphatic duct. The lack of either of these genes affects severely the endolymphatic duct develop-

ment (Depew *et al.*, 1999; Lin *et al*., 2005). The study detected the expressions of *Gbx2* and *Dlx5* in *Gata3–/–* otic vesicles at E9.5, but the level and expression domains were decreased compared to the wt littermates. If two vesicles were present on one side of the head, *Gbx2* was expressed only in the dorsal vesicle and also strong *Dlx5* expression was detectable specifically in the dorsal vesicle (II). These findings further support the observation that D/V polarity is maintained in *Gata3–/–* mutant otic epithelium even after its disruption into two parts.

Additionally, the study compared the expression of other dorsal otic genes, *Wnt2b, Wnt6,* and *Drapc1* (II; Jukkola *et al.*, 2004; Lin *et al.,* 2005), in wt and *Gata3–/–* otic epithelia. While *Wnt2b* expression was clearly detectable in newly closed wt otic vesicles at E9.5, no *Wnt2b* expression was detected in *Gata3–/–* embryos. A remarkable decrease in *Drapc1* expression domain and level was observed at the otic cup stage. The decrease was even more dramatic after otic closure when also *Wnt6* expression decreased strongly, indicating that the expression maintenance of dorsal genes was not fully supported in *Gata3* mutants (II).

Taken together, the present study shows that the expression of several dorsal genes in Gata3 deficient otic epithelium is reduced. The reduced attachment of dorsal otic cup epithelium to the neural tube in *Gata3* mutant embryos might lead to reduction in the received signals emanating from the adjacent hindbrain known to be important for the expression of dorsal otic genes. Particularly, the hindbrain ablation studies by Riccomagno *et al.* (2005) demonstrated that the signals derived from the neural tube, most probably Wnt3a and Wnt1, are responsible for the expression of dorsal otic genes, such as *Gbx2* and *Dlx5*. The expression of *Wnt2b* in the otic vesicle is also thought to be due to hindbrain influence (Lin *et al.,* 2005).

The present study found that in both wt and *Gata3–/–* littermates the ventral expression border of the dorsal genes coincided with the point where the otic placode/cup epithelium loses the attachment to the neural tube. This situation resembles very much the Topgal reporter activity in otic placode and cup epithelium induced by hindbrain derived Wnt's (Riccomagno *et al*., 2005). It is probable that the ventral expression border of the examined dorsal genes is defined by hindbrain-derived signals. Thus, the reduction of the expression domain of dorsal genes in *Gata3* mutant embryos is probably a secondary defect due to reduced amount of hindbrain-derived signal molecules. The fact that *Gata3* is only weakly expressed in dorsal otic cup epithelium is in line with this suggestion. The reduction of dorsal gene expression contributes to the *Gata3–/–* otic phenotype with severely impaired dorsal structures and could explain the aberrant endolymphatic duct development while *Gata3* itself is never expressed there.

Because the axial polarity was established in *Gata3–/–* otic epithelium, and no extension or alteration of ventral gene expression domains was detected (see also 3.3), one cannot consider the reduction of dorsal genes as a general patterning defect. Instead one might propose that the epithelial segregation occurs fairly normally without Gata3.

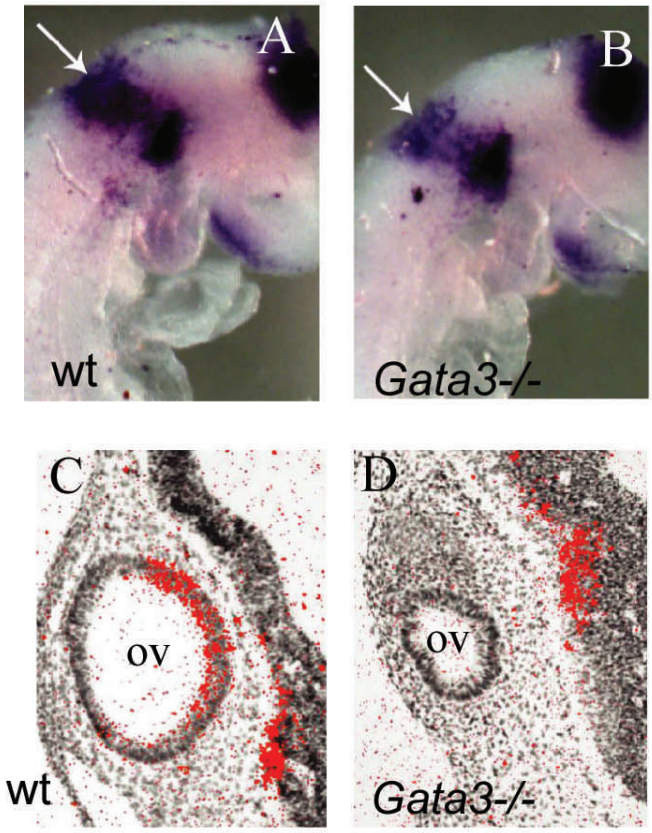

Figure 5. Expression of *Pax8* in wt (A,C) and *Gata3*–/– (B,D) otic region. Wm *in situ* hybridization (A,B) on E8.5 mouse embryos. Radioactive in situ hybridization on paraffin sections (C,D) on E9.5 mouse embryos; the anterior is up, and the medial is right. Arrows in A and B depict the otic placode; ov, otic vesicle.

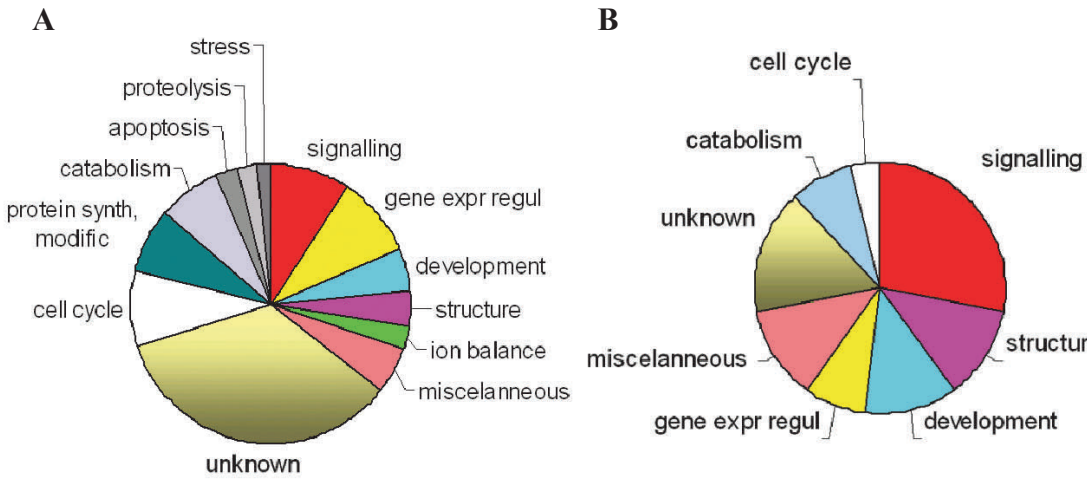

Figure 6. Pie charts indicating the functional gene groups with upregulated (A) and downregulated (B) expression levels in *Gata3*–/– compared to wt E9.5 otic vesicles. Expression level data were obtained from Affymetrix U74v2 Set chipA hybridization analysis. The cut-off limit is 1.7 times and $p < 0.0005$.

3.5. Large-scale gene expression analysis of *Gata3–/–* versus wild type otic epithelium (II)

In order to obtain a better insight into the molecular mechanisms underlying the aberrant early morphogenesis and splitting of the otic epithelium in *Gata3–/–* embryos, a large-scale expression analysis was performed on commercial Affymetrix U74v2 Set; chipA containing 12488 gene probes (including controls).

To obtain technically reliable samples, the newly closed otic vesicles (E9.5) from wt and *Gata3* mutant mouse embryos were dissected as smaller samples of otic placodes or otic cups may have easily been contaminated with surrounding tissues.

Comparison of the cDNA compositions obtained from wt and *Gata3–/–* otic vesicle epithelia revealed a number of misexpressed genes in *Gata3–/–* samples. Surprisingly, the amount of upregulated genes exceeded four times the amount of downregulated genes, indicating that Gata3 is likely to act rather as a repressor than an activator molecule in early otic epithelium. Once the empirical threshold of 1.7 times ($p<0.0005$) was established for the genes, which gave decreased and increased hybridization signals on the chip, there remained 63 and 250 genes for the study, respectively. The set of the obtained genes revealed interesting insight into the *Gata3–/–* early otic phenotype, as discussed below. The marked changes in the expression of the genes that encode factors for the regulation of cell signalling, as well as general factors for gene expression regulation, suggest that most changes occurred as secondary or further downstream effects rather than the primary ones. It was not surprising as the samples were collected a day after *Gata3* expression onset and later than the otic phenotype became detectable.

The misexpressed genes were divided into functional groups, which are presented as pie charts in Figure 6. Detailed data of the Affymetrix hybridization analysis are available as supplementary data I to (II).

Two Gata factors in *Drosophila*, grain and pannier, have been shown to control organ shape by regulating intraepithelial motility and adhesion (Calleja *et al.*, 2000; Brown and Castelli-Gair Hombria, 2000). Similarly, mouse *Gata3* that is expressed throughout the invaginating otic placode, may regulate the intraepithelial properties underlying the phenotype of the *Gata3–/–* mutant.

Indeed, the Affymetrix analysis found several changes in genes that encode proteins involved in mediating cell adhesion (II). Cells are known to respond with their cytoskeleton organization to the changes in adhesion and extracellular matrix compositions (Discher *et al.*, 2006). Accordingly, numerous alterations in the expression levels of the genes that encode cytoskeleton constituents, as well as factors involved in mediating its arrangement and motility, were identified.

One of the biggest alterations (5.7-time increase) was detected in *Gjb2* mRNA level encoding connexin26. RNA *in situ* hybridisation analysis revealed that while in wt embryos *Gjb2* expression was first detected at E10.5 in two sensory patches, then the *Gjb2* gene in *Gata3–/–* otic epithelium was expressed precociously and ectopically already in the otic cup epithelium at E9.25 (II). Connexins form gap junctions between neighbouring cells, thereby mediating the ion flux. Additionally a gap junction-independent role has been established for connexins in mediating cell adhesion and motility (reviewed in Wei *et al*., 2004). Overexpression of *Gjb2* in malignant cells can restore epithelial morphology and reduce cell proliferation, invasion, and migration (Momiyama *et al.,* 2003). Furthermore, the expression level of an ECM encoding gene, *Secreted phosphoprotein 1* (*Spp1*) had decreased four times (II). As the increase of Spp1 is linked to metastasis (reviewed in Wai and Kuo, 2004), its decrease in Gata3 deficient otic epithelium could have an opposite effect. Accordingly, the upregulation of *Gjb2* and downregulation of *Spp1* may lead to increased cellular adhesion and reduced cellular motility.

In conclusion, the detected changes in the expression levels of the genes that encode adhesion mediating molecules, as well as cytoskeleton components and regulators, subsequently lead to altered *Gata3–/–* otic epithelium properties, most probably to reduced intraepithelial cell motility and increased cellular tension.

3.6. Alterations in intraepithelial properties underlie the aberrant otic placode morphogenesis of *Gata3–/–* mouse embryos (II)

Hereby one could propose a hypothetical explanation for the mechanisms behind the aberrant morphogenesis of the otic placode in *Gata3–/–* embryos.

The invagination of *Gata3–/–* mouse otic placodes did not result in as deep and round otic cups as it does in wild type and heterozygous littermates. Differently from uniformly thick and proliferating normal otic cup epithelium, the ectopic morphological boundary often divided the cup epithelium of *Gata3* mutant embryos into a thick ventral domain with several layers of disorganized cells of a high proliferation rate and into a thinner and less proliferating dorsal domain. Interestingly, the ectopic dorso-ventral morphological boundary along the medial wall of the *Gata3–/–* otic epithelium coincided with the ventral expression border of several dorsally expressed genes and with the loss of the attachment between the neural tube and the otic epithelium. Thus it is likely that the expression of *Gata3* is required for permitting the highly proliferating ventral cells to contribute normally to the dorsal otic cup compartment across the border that could be determined by the neural tube derived signals. The described situation causes the appearance of precocious endolymphatic cha-

racteristics, such as reduced proliferation and a thin epithelial structure. In the most severe cases the endolymphatic domain separates from the rest of the otic epithelium.

The proposed explanation takes into account the possibility of reduced motility due to increased adhesion (Chapter 3.5) and the data from the fate-mapping study by Brigande *et al*. (2000a) who showed that in the chicken the otic cup closure is achieved by extensive cellular rearrangement and that the cells originating from the postero-ventral domain expand most extensively.

3.7. Gata3 is specifically required for the differentiation of auditory sensory epithelia (I, II, and unpublished data)

Gata3 is expressed throughout the early otic epithelium, encompassing the prosensory domain. Later, *Gata3* is expressed in all sensory epithelia with the only exception of saccular macula. In vestibular sensory epithelia *Gata3* becomes downregulated after E14.5, and the only sensory region where the expression persists throughout the development including postnatal stages is the striolar region of utricular macula (I; Karis *et al*., 2001; Lawoko-Kerali *et al*., 2002). Unlike vestibular sensory epithelia, *Gata3* expression in auditory prosensory epithelium persisted without downregulation in postmitotic hair and supporting cells (I; van der Wees *et al*., 2004).

The expression of *Gata3* in the prosensory epithelium region and in the course of sensory patch development might indicate a role in sensory determination and/or differentiation. Thus the study examined the expression of several early sensory marker genes as well as the markers for postmitotic hair and supporting cells in *Gata3–/–* otic epithelium.

The known early marker genes for prosensory mouse otic epithelium include *Lfng, Bmp4,* and *Jag1*. In the newly closed otic vesicle *Lfng* and *Jag1* are expressed in the antero-ventral portion of the otic epithelium. After separation of distinct sensory epithelium primordia (occurs between E10.5–13.0) *Lfng* becomes confined to developing maculae and the organ of Corti while *Jag1* is expressed in each of the prosensory domains (Adam *et al.*, 1998; Morsli *et al.,* 1998; Morrison *et al.,* 1999). The expression of *Bmp4* becomes confined to two separate domains at E10.5, from which the posterior domain develops into the posterior crista, and the anterior domain produces the anterior and lateral cristae (Morsli *et al.,* 1998).

Similar expression patterns of *Lfng*, *Bmp4,* and *Jag1* were detected in the early *Gata3–/–* otic epithelium, such as in wt embryos (II and unpublished data). These results indicate that Gata3 is not required for establishing sensory fate in the early otic epithelium.

Also the expression of *Fgf10*, the sensory and neural specific gene in mouse otic epithelium, was checked. Surprisingly, the study found that in contrast to

wt littermates, the expression of *Fgf10* was not initiated in *Gata3–/–* otic epithelium (II). Nevertheless, the absence of Fgf10 in Gata3 deficient otic epithelium cannot interfere with sensory development as sensory epithelia form and differentiate in *Fgf10* deficient mice, except the posterior crista, which fails to form (Pauley *et al*., 2003).

In order to study the later sensory differentiation in *Gata3–/–* embryos drug treatment (Kaufman *et al.*, 2003) was used to rescue the *Gata3–/–* mouse embryos that survived up to E16.5–18.5.

Jag1 is continuously expressed throughout the development of the sensory epithelium, being confined to supporting cells by E17.5 (Morrison *et al*., 1999). The expression of *Jag1* was detected in all sensory epithelia in wt embryos at E11.5–E15.5 (unpublished data; Figure 7A,C,I). *Jag1* was strongly expressed also in *Gata3–/–* otic epithelium at the respective stages (unpublished data; Figure 7B,D,J). Interestingly, the expression region of *Jag1* was proportionally much broader in mutants than in wt littermates (unpublished data; Figure 7A,B,I,J). This finding indicates that the prosensory epithelium development might be less disturbed compared to non-sensory epithelium in *Gata3* mutants.

In mice, the hair and supporting cell progenitors become postmitotic between E12.5–E14.5 (Ruben, 1967). To study further differentiation of hair and supporting cells, the study analysed the expression of a cell cycle inhibitor $p27^{Kip1}$, which becomes detectable in postmitotic hair and supporting cell progenitors (Chen *et al*., 2002). $p27^{Kip1}$ expression in the wt organ of Corti was detected at E14.5 (unpublished data; Figure 7E). By contrast, no simultaneous $p27^{Kip1}$ expression was detectable in *Gata3–/–* cochlea whereas the expression was observed in the vestibular sensory areas (unpublished data; Figure 7F).

Mouse bHLH transcription factor Math1 is essential for hair cell specification, and *Math1* mutant mice do not develop hair cells (Bermingham *et al*., 1999). *Math1* expression is initiated at E12.5 in the vestibular and at E13.5 in the cochlear sensory epithelium. The expression of *Math1* starts both in hair and supporting cell progenitors, but it later remains restricted specifically to hair cell progenitors and mature hair cells (Chen *et al*., 2002; Woods *et al*., 2004).

Accordingly, the study detected *Math1* in all wt sensory areas at E14.5–15.5 (unpublished data; Figure 7G). Also, weak *Math1* expression was observed in the vestibular sensory epithelia of *Gata3–/–* ears, but no expression was detected in the cochlear domain (unpublished data; Figure 7H,K,L).

The morphology of the ears was dramatically affected in *Gata3* mutants making it often impossible to distinguish the different ear regions. In most serious cases, the otic epithelium formed only a single otic cavity with no clear compartments. In these samples the expression of *Jag1* and *Math1* was compared in adjacent sections. It appeared that only a restricted part of the dorsal *Jag1* positive domain was able to differentiate further and initiate *Math1* expression (Figure 7J,K). This Math1 positive domain was most probably determined to develop into vestibular sensory epithelia.

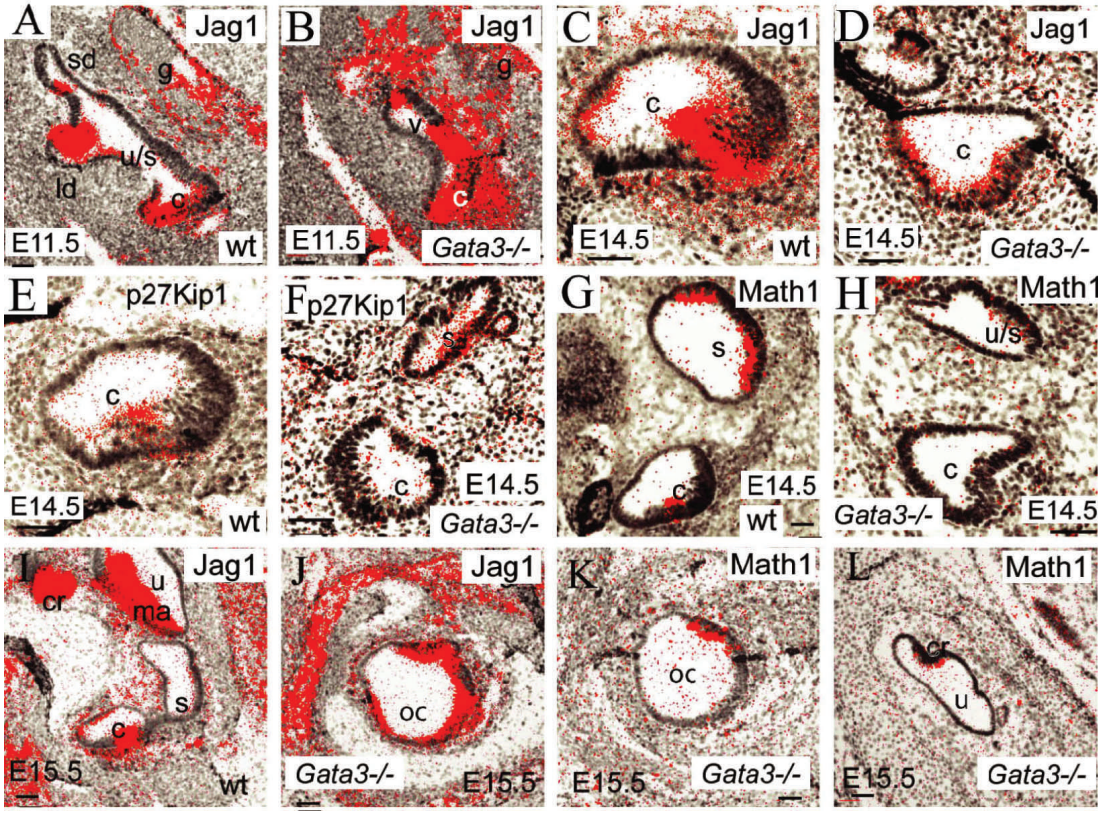

Figure 7. Sensory differentiation in *Gata3*–/– otic epithelium. Expression analysis on serial paraffin sections from wild type (A,C,E,G,I) and *Gata3*–/– (B,D,F,H,J,K,L) embryos at E11.5 (A,B), E14.5 (C-H), E15.5 (I-L). The dorsal is to the right, and the anterior is up. Probes are shown in the images. sd, superior semicircular duct; ld, lateral semicircular duct; c, cochlea; s, saccule; u, utricle; u/s, utriculo-saccular area; ma, macula; cr, crista; oc, otic cavity; v, vestibular area. Scale bar is 50 µm.

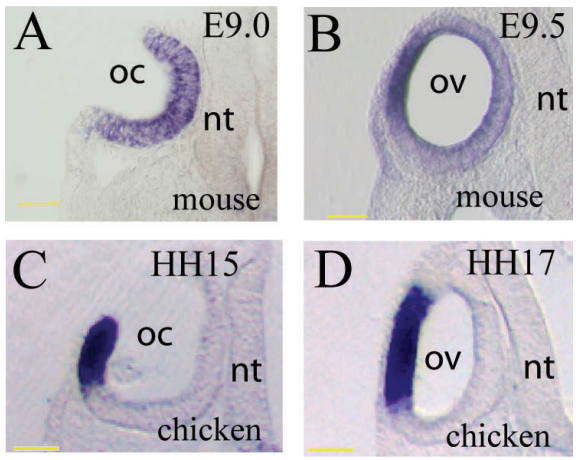

Figure 8. Expression of *Gata3* before and after otic closure in mouse (A,B) and chicken (C,D). Whole-mount *in situ* hybridisation. Vibratome sections. The dorsal is up, and the medial is right. Stages are shown in images. nt, neural tube; oc, otic cup; ov, otic vesicle. Scale bar is 50 µm.

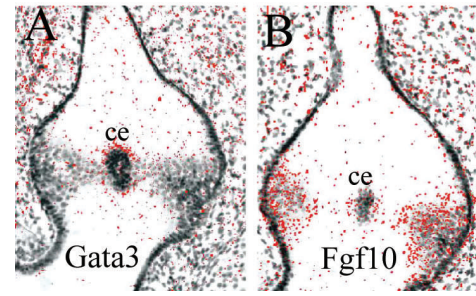

Figure 9. Expression of *Gata3* and *Fgf10* in mouse superior crista at E14.5. Radioactive *in situ* hybridization on paraffin sections. ce, cruciate eminence.

Brn3.1 expression follows *Math1* and is required for terminal hair cell differentiation (Erkman *et al*., 1996; Xiang *et al*., 1997; Xiang *et al*., 1998). *Brn3.1* expression was detected both in vestibular and cochlear sensory epithelia of the E15.5 wt embryos but similarly to $p27^{Kip1}$ and *Math1* only in dorsal sensory patches of *Gata3–/–* embryos (unpublished data).

To sum, the findings of this study show that the inner ear sensory primordium is established in the absence of Gata3 and that the Gata3 deficient vestibular sensory epithelia are able to produce postmitotic progenitors of hair and supporting cells. However, the cochlear prosensory epithelium of the *Gata3–/–* embryos remains immature and fails to differentiate into postmitotic hair and supporting cell progenitors, which shows that Gata3 has a specific role in the development of auditory sensory epithelium. Tissue culture experiments have shown that the surrounding mesenchyme has an important role in the differentiation and patterning of the organ of Corti (Montcouquiol and Kelley, 2003). Since *Gata3* is expressed both in the prosensory epithelium and in cochlear mesenchyme (I), it could play a role in auditory sensory differentiation either cell-autonomously and/or by regulating mesenchyme-derived signals.

3.8. Fgf-signalling is altered in *Gata3–/–* otic epithelium (II)

Fgf-signalling is involved in ear induction, morphogenesis, and hair cell development (Mansour *et al*., 1993; Alvarez *et al*., 2003; Pauley *et al*., 2003; Wright and Mansour, 2003). In the mouse, mesenchyme-derived Fgf10 and hindbrain-derived Fgf3 act redundantly to induce the otic placode. In the otic epithelium, the expressions of *Fgf10* and *Fgf3* are initiated at the otic cup stage (Alvarez *et al*., 2003). While the early otic development is affected neither in *Fgf10* nor in *Fgf3* mutant embryos, then the embryos lacking both genes develop severely reduced otic placodes and vesicles (Alvarez *et al*., 2003; Wright and Mansour, 2003).

In newly closed wt otic vesicles a partial overlap of *Fgf10* and *Fgf3* expression domains in the antero-ventral region and in the cells forming the vestibulocochlear ganglion was observed. Unlike wt littermates, *Fgf10* was not expressed in *Gata3–/–* otic cups and vesicles. The expression of *Fgf3* was unchanged in *Gata3–/–* otic epithelium indicating that the loss of Fgf10 was not caused by alterations in the identity of the otic region where *Fgf3* and *Fgf10* are expressed together and that Gata3 is specifically required for *Fgf10* expression (II).

In chickens the early expression of *Fgf10* is known to promote neural fate via positive regulation of *NeuroD/NeuroM* (Alsina *et al*., 2004). The normal neural development of *Fgf10* mutant mouse embryos suggests that another member of the Fgf family, for example Fgf3, may substitute Fgf10 in early otic

epithelium. Nevertheless, as the expression of *NeuroD* has been shown to be under the positive regulation of Gata3 in mouse migrating auditory neuroblasts (Lawoko-Kerali *et al*., 2004) and the microarray analyses of this study showed that *NeuroD* mRNA level had clearly decreased already in newly closed *Gata3–/–* otic vesicle epithelium at E9.5 (II), one can deduce from the findings of this study that Gata3 could regulate *NeuroD* expression at least partially through Fgf10-signalling.

During semicircular duct outgrowth *Gata3* and *Fgf10* are both expressed in the vestibular sensory domains (I; Lawoko-Kerali *et al*., 2002; Pauley *et al*., 2003). It has been proposed that Fgf10, secreted from the sensory epithelium, directs the outgrowth of the semicircular ducts in chicken embryos by up-regulating *Bmp2* in the duct genesis zone (Chang *et al*., 2004). Accordingly, mice with mutated *Fgf10* develop severely reduced semicircular ducts (Pauley *et al.,* 2003). Thus, the loss of Fgf10 from the vestibular sensory epithelia should contribute to the lack of semicircular duct outgrowth in *Gata3* deficient embryos. However, as noted, the reduced contribution of ventral cells to the dorsal compartment of the early otic epithelium and reduced expression of dorsal genes are probably the main causes of the phenotype generation of the semicircular duct in *Gata3–/–* embryos that is indeed more severe than in *Fgf10–/–* embryos.

3.8.1. Gata3 is a direct regulator of *Fgf10* (II)

As *Fgf10* expression was not initiated in Gata3 deficient otic epithelium and the expression domains of *Fgf10* and *Gata3* overlap considerably in the early otic epithelium and later in the developing ganglion and sensory epithelia, the study focused on the ability of Gata3 to regulate the expression of *Fgf10* directly.

Using Consite algorithm (Lenhard *et al*., 2003) it was found that *Fgf10* upstream region contains several Gata3 binding sites, which are conserved between the mouse, the human and the chicken (II). A 3410 bp upstream region containing the conserved Gata3 binding sites from *Fgf10* was cloned into a firefly luciferase reporter vector (pGL3-Basic). The resulting construct pGL3-*Fgf10(–3410)* was transfected into NIH3T3 cells with or without a Gata3 expression vector. The choice for the used NIH3T3 cells was made due to the endogenous expression of *Fgf10* indicating the presence of the required factors (II).

As a result, the full *Fgf10(–3410)* genomic region together with Gata3 overexpression was able to transactivate the reporter gene approximately twofold. Although the observed effect was statistically highly relevant, the relatively low transactivation rate was probably caused by the endogenous expression of *Gata3* in the used NIH3T3 cells (II).

Deletion analysis mapped the Gata3 binding sites containing region responsible for reporter gene transactivation between nucleotides –3410

to –1659 from the transcription start site. More precisely, the conserved Gata3 binding sites remained between the nucleotides –3335 to –2547 (Supplementary data 2 in II).

Taken together, the findings of the study strongly suggest that *Fgf10* is a direct target for Gata3 in mouse otic epithelium. No other direct targets for Gata3 in the inner ear have been identified before, and Gata3 is the first factor discovered to regulate directly *Fgf10* expression in the inner ear. A recent study by Ohuchi *et al*. (2005) is in line with the findings of this study, demonstrating that the same upstream region is responsible for ear-specific *Fgf10* expression.

3.9. Early otic development occurs normally in *Gata2–/–* embryos (I, II, and unpublished data)

A close relative to *Gata3*, *Gata2* was also expressed in mouse inner ear during embryogenesis. *Gata2* expression was initiated in the lateral wall of the newly closed otic vesicle at E9.5 and became restricted to the dorso-lateral and ventro-medial compartments at E10.5 in a manner that is similar to *Gata3* (I). Since nothing was known about the role of Gata2 in mouse inner ear, the otic vesicle formation was examined in *Gata2–/–* mouse embryos. No morphological defects in Gata2 deficient otic vesicles were detected at E10.5 when the mutants die (I).

In order to figure out whether the otic epithelium patterning is preserved in otic vesicle epithelium that would lead to defects later, the expression domains of ventro-medial *Pax2,* ventral *Eya1, Six1*, and dorsal *Dlx5* were checked. It appeared that the otic vesicle polarity was established normally in *Gata2–/–* embryos. Using *Bmp4* as a marker for presumptive cristae, it was shown that also sensory determination occurred normally without Gata2 (I).

In order to obtain a better insight into the later role(s) of Gata2 during inner ear development a conditionally inactivated *Gata2* mouse line was created and a relatively late phenotype was observed, which was obvious at E15.5–16.5 and characterized by smaller semicircular ducts (unpublished data).

Gata3 and Gata2 have been shown to bind to similar DNA sites (Ko and Engel, 1993). Since in the otic vesicle the expression patterns of *Gata2* and *Gata3* were highly overlapping, and *Gata3* was expressed normally in Gata2 deficient otic vesicles (I), it is probable that Gata3 may substitute Gata2 in early otic epithelium. Moreover, in conditionally mutated *Gata2–/–* embryos the aberrant otic phenotype coincided with the downregulation of *Gata3* expression from the semicircular duct epithelium. Redundancy between these factors has been suggested also in inner ear efferent neurons where Gata2 may temporarily substitute Gata3 (Pata *et al.,* 1999).

The expression of *Gata3* is controlled by Gata2 in developing mouse hindbrain (Nardelli *et al*., 1999; Pata *et al*., 1999). The present study showed an

opposite situation in the ear, where *Gata2* expression started at the newly closed otic vesicle (E9.5), that is later than *Gata3* expression at the placodal stage (I, II). Moreover, the present study showed that Gata3 deficient otic vesicles exhibit a strong delay and decrease in *Gata2* expression but not *vice versa* (I). These results indicate that in the ear Gata2 and Gata3 may act in the same pathway as in the hindbrain, but in the reverse order.

3.10. Comparative analysis of *Gata3*, *Gata2*, and *Fgf10* expression during mouse and chicken inner ear development

The present study described in detail the expression patterns of *Gata3* and *Gata2* during mouse and chicken inner ear development. The focus was on i) obtaining a better insight into the potential roles of these factors during the formation of the membranous labyrinth; ii) the possible conservation of the Gata3 and Gata2 regulated events between mammals and birds. The study also aimed at finding out whether Gata3 could regulate the expression of *Fgf10* and *Gata2* in chicken, as is the case with mice. For this purpose the expression of *Fgf10* was followed also during chicken ear development.

3.10.1. Differences in the expression patterns of mouse and chicken *Gata3* suggest a non-conserved molecular control in early otic morphogenesis (I, II, III, and unpublished data)

Mouse *Gata3 (mGata3)* expression was initiated in the otic placode epithelium at E8.5. During invagination it was expressed throughout the otic epithelium, but the strongest expression appeared in the medial region of the otic cup close to the neural tube (II; unpublished data; Figure 8A). The data of the present study indicated that early *mGata3* expression could be involved in the precise cellular rearrangement required for correct otic invagination.

Chicken *Gata3 (cGata3)* was strongly expressed already at the preplacodal stage (HH9) covering a broad domain of the surface ectoderm including the future otic region. A similar expression persisted until HH12 when the otic placode starts to invaginate (III). The preplacodal *cGata3* expression suggests that the chicken factor may play even an earlier role in ear development than the mouse counterpart. In the otic cup epithelium (HH14–15) *cGata3* expression became restricted to the antero-lateral domain including the lateral rim of the closing otic epithelium (III; Figure 8C).

Thus, the distribution of *Gata3* transcripts in invaginating otic epithelium differs remarkably between mouse and chicken (Figure 8A,C). The absence of

cGata3 expression from the medial otic cup epithelium suggests that the chicken factor is not involved in the regulation of cell motility and epithelial morphology in the medial wall as is the case with mice.

After otic closure, *Gata3* was expressed in a similar manner both in the mouse and the chicken. Particularly, *cGata3* remained restricted to the antero-lateral wall after otic closure at HH17 (III; Figure 8D). Also, the strongest *mGata3* domain had shifted laterally in newly closed (E9.5) mouse otic vesicles (I; II; Figure 8B). The otic vesicle starts to elongate at E10.5 in the mouse and at HH19 in the chicken. At this stage the expression of *mGata3* became restricted to two separate regions, the dorso-lateral and the ventro-medial regions, and strong expression appeared also in periotic mesenchyme adjacent to the epithelium expressing *mGata3* (I). Similarly, at HH19 *cGata3* expression appeared also ventro-medially in addition to a broad lateral expression domain and about half a day later (at HH21) also in distinct dorsal and ventral domains of the periotic mesenchyme (III).

The observation that the expression patterns of *Gata3* in invaginating otic epithelium and in the otic vesicle exhibit different conservation rates suggests at least two independent roles for Gata3 in early otic epithelium. While the involvement of Gata3 in the otic placode morphogenesis is not conserved between mouse and chicken, the conserved strong lateral expression in the closed otic vesicle points to a common role in the development of the derivatives of the lateral wall, such as the semicircular ducts, which are indeed missing in Gata3 deficient embryos. Furthermore, the results of the present study suggest that the molecular control over the earliest morphogenetic step when the otic placode forms into a vesicle may not be conserved between mammals and birds.

3.10.2. Redundancy and hierarchy between *Gata2* and *Gata3* could be conserved in evolution (I and III)

As described in Chapter 3.9, mouse *Gata2 (mGata2)* expression overlapped highly with *mGata3* expression domains in otic vesicle epithelium. The data of the present study strongly suggest a functional redundancy between these factors. Additionally, the expression of *mGata2* was remarkably reduced and delayed in Gata3 deficient otic vesicles (I).

Similarly to mouse counterparts, the expression onset of chicken *Gata2 (cGata2)* followed *cGata3,* and their expression domains overlapped remarkably in early otic epithelium. Particularly, the expression of *cGata2* started at the late otic cup stage HH15 in a small ventro-lateral domain, which was completely included in the broader *cGata3* expression domain. In the closed otic vesicle (HH17, HH19) *cGata2* continued to be expressed in the lateral wall, overlapping with *cGata3* expression domain (III). The highly

overlapping expression domains of *cGata2* and *cGata3* in early otic epithelium make the functional redundancy, as shown in the mouse, possible also in chicken. The temporal order of the chicken *Gata* gene expression onset suggests conservation also in their regulatory hierarchy.

3.10.3. *Fgf10* could be a target for Gata3 also in chicken inner ear (II and III)

Mouse *Fgf10 (mFgf10)* expression was first detected in the anterior part of the invaginating otic cup; in the newly closed otic vesicle it continued in the antero-ventral epithelium (Alvarez *et al*., 2003; Pauley *et al*., 2003). At these early stages, *mFgf10* transcripts were found inside the broader *mGata3* expression domain. It appeared that mGata3 is responsible for the initiation of *mFgf10* expression in the otic epithelium and developing ganglion probably via direct interaction with an upstream element of *mFgf10* gene, which consists of several Gata3 binding sites that are conserved between the mouse, the human and the chicken (II). Thus, a similar regulation may occur also in chicken otic epithelium.

In order to find the extent of overlap in their expression domains of chicken *Fgf10 (cFgf10)* and *cGata3*, their early expression patterns were compared. Similarly to the mouse, *Gata3* expression preceded *Fgf10* expression in chicken otic epithelium. *cFgf10* was initiated at the anterior pole of the otic placode around HH11–12, being included in a broad *cGata3* domain. At subsequent otic cup and vesicle stages, *cFgf10* expression extended more medially with only a minor overlap with the laterally distributed *cGata3* expression domain (III). These data suggest that the initiation of *Fgf10* expression in chicken otic epithelium might be dependent on Gata3 as was shown in the case of mouse counterparts.

3.10.4. Expression studies in the vestibule show partial conservation of *Gata3*, *Gata2* and *Fgf10* expressions between mammals and birds

In the mouse, the period between E11.5–14.5 is a time of rapid morphogenesis when all the different inner ear structures develop from a simple elongated otic vesicle (Martin and Swanson, 1993). The corresponding morphogenetic period in the chicken occurs between HH23–30 (Bissonnette and Fekete, 1996). The utricle and the saccule become separated from each other by constriction in the ventral vestibule while the three semicircular ducts are formed via a multi-step process in the dorsal portion (Martin and Swanson, 1993; Bissonnette and Fekete, 1996; Morsli *et al*., 1998). During this period, also the hair cells in

different sensory epithelia start to differentiate (Goodyear *et al*., 1995; Chen *et al*., 2002).

3.10.4.1. Expression of *Fgf10* (III and unpublished data)

mFgf10 expression is confined to sensory epithelia and sensory neurons during otic development (Pirvola *et al*., 2000; Pauley *et al*., 2003). Respectively, *mFgf10* expression was detected in the development of maculae and cristae at E12.5–E18.5. In cristae *mFgf10* was expressed all through the sensory organ, including non-sensory cruciate eminence and the hair cell region (unpublished data; Figure 9B; Pauley *et al*., 2003). The *Fgf10* mutant mice display malformations in the semicircular duct system; the posterior duct and its crista are completely absent and in the severely disturbed lateral and anterior ducts the cristae form without cruciate eminences (Pauley *et al*., 2003).

While in early otic epithelium the expression domain of chicken *Fgf10* had been confirmed to the region possessing sensory and neural competence (Alsina *et al*., 2004), its later expression during chicken otic development was largely unknown. In order to verify *cFgf10* distribution in developing sensory epithelia its expression was compared with chicken *Bmp4* expression, the marker for all of the chicken sensory epithelia (Wu and Oh, 1996). The present study showed in HH24–HH38 chicken that in accordance with the mouse, *cFgf10* was expressed in developing maculae and cristae. As a difference compared to mouse, the expression of *cFgf10* in chicken cristae was restricted to the sensory portion of the newly formed crista ampullaris and was excluded from the lingula parts, which were devoid of hair cells (III).

In chicken the expression of *cFgf10* in presumptive cristae has been shown to regulate positively *Bmp2* expression in the genesis zone of the semicircular duct, which is important in the outgrowth of semicircular ducts (Chang *et al*., 2004). While the role of Fgf10 in the formation of the semicircular ducts could be conserved both in the mouse and the chicken, then the expression of *mFgf10* in cruciate eminence and the lack of *cFgf10* from the lingula parts of the cristae could indicate different molecular regulation in the development of the non-sensory compartment of the crista ampullaris in mammals and birds.

3.10.4.2. Expression of *Gata3* (I, III, and unpublished data)

During vestibular development *Gata3* was expressed in the epithelium and surrounding mesenchyme both in the mouse and the chicken. *mGata3* expression was progressively downregulated from the vestibular epithelium and at E14.5 its expression was restricted to prosensory epithelia with the only exception of saccular macula (I; Karis *et al*., 2001). Similarly to the mouse, *cGata3* expression was also detected in the development of vestibular sensory epithelia. However, unlike the mouse, much broader and persistent *cGata3*

expression was detectable in the vestibular non-sensory epithelium of the chicken.

In outgrowing semicircular ducts Gata3 expression was confined to the distal parts in a similar manner both in the mouse and the chicken (I; III). Being expressed also in periotic vestibular mesenchyme, Gata3 might possess a conserved role in controlling the semicircular ducts morphogenesis via regulation of mesenchymal-epithelial signalling.

In utricular macula *Gata3* became confined to the striolar region in both species (unpublished data; III; Karis *et al.,* 2001; Hawkins *et al*., 2003; Warchol and Speck, 2007). The striola is a crescent-shaped narrow area that divides the macula into two parts with opposite hair cell polarity (Figure 3C; Denman-Johnson and Forge, 1999). Therefore, Gata3 could possess a conserved role in the determination of hair cell polarity.

In maturating cristae *Gata3* expression became restricted specifically to the non-sensory parts – the cruciate eminence in mouse embryos (unpublished data; Figure 9A; Karis *et al*., 2001) and to the lingula parts of chicken cristae (III). The overlapping expression of *mGata3* and *mFgf10* in cruciate eminence suggests that mGata3 may influence the formation of this structure by controlling *mFgf10* expression. Although cGata3 might regulate *cFgf10* expression early in presumptive cristae regions of the chicken, the strictly complementary expression in more mature cristae excludes the possibility that cGata3 is directly required for the maintenance of *cFgf10* expression.

A restricted and persistent *Gata3* expression domain existed in a small non-sensory region of the vestibular epithelium residing between the utricular macula and the lateral crista both in the chicken and the mouse (III). This region seems special since it is the only non-sensory domain that is clonally related to the neurons in the cochlear ganglion (Satoh and Fekete, 2005). Thus *Gata3*, being specifically expressed also in the cochlear ganglion, can be considered a marker for these clonally related tissues.

In conclusion, the expression data suggest that in the non-sensory epithelium Gata3 could play a more important role in chicken compared to mouse. The expression of *Gata3* in prosensory vestibular epithelia and later the restricted and highly conserved expression pattern of *Gata3* in specific parts of vestibular sensory organs indicate a conserved role in the development of these organs. However, the particular requirement for *Gata3* expression in developing sensory organs remains elusive as in the mouse the sensory cells were able to differentiate without Gata3 and sometimes also recognizable cristae formed (unpublished data; Figure 7L).

3.10.4.3. Expression of *Gata2* (I, II, and unpublished data)

The expression of *Gata2* was predominant and continuous in vestibular non-sensory epithelium both in the mouse and the chicken. *mGata2* was strongly expressed all over the developing semicircular duct epithelium, and its expression was detectable also in the adjacent mesenchyme (I). The present study with conditionally inactivated *mGata2* mouse line revealed that in the mouse the semicircular ducts form without mGata2, and no clear phenotype could be detected before E14.5. However, at E15.5–E16.5 all three semicircular ducts were reduced in diameter, and the cells surrounding the ducts were not sufficiently removed to generate the perilymphatic space (unpublished data).

Unlike the mouse, in developing chicken semicircular ducts *cGata2* expression was complementary to *cGata3*, and it was restricted to the proximal areas that contribute to the fusion plates (III). *cGata2* was expressed also in the neighbouring periotic mesenchyme (III), known to participate in fusion plate formation (Salminen *et al*., 2000). Thus, unlike the mouse, the chicken Gata2 could regulate the fusion event during the formation of semicircular ducts.

In contrast to *Gata3*, *Gata2* was not expressed in any of the vestibular sensory epithelia in either of the species, indicating that it is not directly involved in vestibular sensory development. *Gata2* expression was detected in the fibrocytes underlying the vestibular sensory epithelia in both the mouse and the chicken (I; III). These specialized cells regulate inner ear fluid movement and ion homeostasis essential for proper hair cell functioning (Delprat *et al*., 2002). Thus, Gata2 may control the development and/or survival of the fibrocytes. *Gata2* expression was detectable also in the dark cell region in both species (III; unpublished data), which points to a role in endolymph production. The reduced endolymph production could underlie the reduced semicircular duct phenotype, as it is thought to be in the case of *EphB2* mutant mice (Cowan *et al.*, 2000). The failure in endolymph production and/or homeostasis might indirectly influence sensory development.

3.10.5. Expression patterns of *Gata3, Gata2,* and *Fgf10* in cochlea exhibit a high conservation rate (I, II, III, and unpublished data)

The cochlear duct starts to grow out from the ventral part of the otic vesicle at E11.5 in the mouse and at HH23 in the chicken. The lateral cochlear duct epithelium is thinner and develops non-sensory structures, including the stria vascularis in the mouse and the tegmentum vasculosum in the chicken while the thicker medial epithelium contributes to the sensory organs – the organ of Corti in the mouse and the basilar papilla in the chicken (Martin and Swanson, 1993; Bissonnete and Fekete, 1996; Rinkwitz *et al*., 2001; reviewed in Kiernan *et al*., 2002).

The expression pattern of *Gata3* was similar in the growing cochlear duct of the mouse and the chicken – expression was detectable in the prosensory medial wall and a weaker expression in the thin lateral wall (I; III). In the course of the developmental progress *Gata3* was strongly expressed in the hair and the supporting cells of auditory sensory epithelium in both species. This expression persists also later in postnatal/posthatch animals (III; Hawkins *et al.,* 2003; van der Wees, 2004).

The strong and constant expression of *Gata3* in the cochlear sensory organs of the mouse and the chicken could be involved in the differentiation of the auditory sensory epithelium and/or in hair cell maintenance. Chapter 3.7 showed convincingly that Gata3 is specifically involved in the differentiation of auditory sensory epithelium. However, since the role of Gata3 in mesenchyme is unknown during the differentiation of auditory sensory epithelium, it remains unclear whether Gata3 is required for hair and supporting cell differentiation cell-autonomously or indirectly via regulating mesenchymal signals. Additionally, impaired hair cell maintenance leading to hearing loss has been demonstrated in *Gata3*+/– mice (van der Wees *et al*., 2004). Nevertheless, as *Gata3* expression was detected also in mouse stria vascularis and in its equivalent tegmentum vasculosum in the chicken (I; III), the structure responsible for endolymph production, it could be also possible that the hair cell maintenance in *Gata3* heterozygous mice, as well as in HDR patients, is disturbed due to failure in endolymph homeostasis.

It was shown that in early otic epithelium the lack of *Gata3* expression causes precocious and ectopic *Gjb2* expression (II). *Gjb2* is expressed in stria vascularis and its alterations are a major cause of human hereditary deafness (Kudo *et al*., 2003). In order to understand whether changes in *Gjb2* expression could be responsible for hearing loss caused by *Gata3* haploinsufficiency, *Gjb2* expression was verified in *Gata3*+/– mouse cochleae at E17.5. No differences in *Gjb2* expression between wt and *Gata3*+/– striae vasculares were detected (unpublished data). This observation suggests that alterations of *Gjb2* expression do not cause the hearing defects in *Gata3*+/– mice.

Also, the *Gata2* mRNA distribution was similar in the cochlear duct of the mouse and the chicken, being detectable both in the prosensory medial and nonsensory lateral walls exhibiting partial overlap with the *Gata3* expression domain (I; III). Thus, unlike the vestibular system, *Gata2* may have a direct role in early cochlear sensory development. *Gata2* was downregulated from maturating auditory sensory epithelium so that no signal was detected in differentiated auditory hair cells in either of the species. It implies that Gata2 is unlikely to be involved directly in auditory hair cell maintenance. As with *Gata3*, *Gata2* that is expressed in the stria vascularis in mouse and the tegmentum vasculosum in chicken (I; III) may be responsible for hair cell maintenance via regulating endolymph production and homeostasis.

In growing mouse cochlear duct *Fgf10* expression becomes confined to the prosensory epithelium. During cochlear maturation *mFgf10* was expressed

strongly in the greater epithelial ridge (GER) and weakly in the hair cell region (Pauley *et al*., 2003 and unpublished data). *mFgf10* is downregulated from the hair cells during the first postnatal weeks (Pirvola *et al*., 2000).

Similarly, the expression of c*Fgf10* was restricted to the thick prosensory domain of the growing cochlear duct in the chicken at HH24–28, overlapping partially with the *Gata3* expression domain. At HH38 strong *cFgf10* expression was observed in the hair cell region (III), but it is not known whether its downregulation happens in the developmental progress as is the case with the mouse. Thus, the findings of the present study suggest that at least the early role of Fgf10 signalling in auditory sensory development is conserved between mammals and birds. Nevertheless, *Fgf10* mutant mice do not exhibit any cochlear defects (Pauley *et al*., 2003), suggesting that other members of the Fgf family, for example Fgf9 (Colvin *et al*., 1999) may play redundant role(s). Nothing is known about the expression of other Fgf family members in chicken basilar papilla as yet.

3.10.6. Expression patterns of *Gata3*, *Gata2,* and *Fgf10* in mouse and chicken otic ganglion (I, II, and III)

The common sensorineural competent region is specified already in the otic placode in the chicken and in the otic cup epithelium in the mouse (Adam *et al*., 1998; reviewed in Rubel and Fritzsch, 2002). The specified neuroblasts start to migrate out from the otic epithelium to form the vestibulo-acoustic ganglion during the cup stage in the chicken (Hemond and Morest, 1991) and at the early vesicle stage in the mouse (reviewed in Fritzsch, 2003).

In both species *Fgf10* expression domain is known to mark the region possessing sensory and neural competence, and *mFgf10* is known to be continuously expressed throughout the neural development both in the developing vestibular and cochlear neurons (Pirvola *et al*., 2000; Alsina *et al*., 2004). While in the chicken the early role of cFgf10 has been established to promote neural fate by regulating positively the proneural and neurogenic genes, the role of mFgf10 remains unknown since no ganglionic phenotype has been observed in *mFgf10* mutants (Alsina *et al*., 2004; Pauley *et al*., 2004). In the case of the mouse, other family members could substitute for mFgf10 during otic ganglion formation. For example, *mFgf3* is expressed together with *mFgf10* in neuronal precursors (Pirvola *et al*., 2000), and has been shown to act redundantly also for otic vesicle formation (Alvarez *et al.,* 2003).

Similarly to the mouse, the present study detected continuous *cFgf10* expression in migrating neuroblasts and in the ganglionic neurons. Considerable upregulation of *cFgf10* in chicken otic ganglion occurred at HH26 (III), coinciding with the generation of postmitotic neurons. This observation

suggests a specific role for cFgf10 in neuronal differentiation and/or maintenance.

As noted, *Gata3* is expressed throughout the early otic epithelium encompassing the *Fgf10* expressing neurogenic region both in the mouse and the chicken (II; III; Pirvola *et al*., 2000; Alsina *et al*., 2004). In the mouse, continuous *mGata3* expression persisted in a subset of delaminating and migrating neuroblasts, which are thought to be associated specifically with the auditory neuron precursors. In fact, mGata3 is considered to be required for the maintenance of *NeuroD* expression in migrating auditory neuroblasts (Lawoko-Kerali *et al.,* 2004). In *Gata3–/–* mouse embryos, the sensory neuron formation is reduced either directly due to failure in auditory neuron development or secondarily through the reduced size of the otic epithelium (Karis *et al*., 2001).

cGata3 expression became restricted to the lateral part of the otic cup during otic placode invagination, only marginally overlapping with the more medial *cFgf10* expressing neurogenic area. In the late otic vesicle stage at HH19–21, strong *cGata3* expression appeared in the antero-ventral epithelium from which the neuroblasts migrate out (III), suggesting that also cGata3 may be involved in auditory neuron specification. In contrast to *mGata3*, *cGata3* mRNA was not detectable in migrating neuroblasts (III), indicating that *NeuroD* maintenance in chicken auditory neuroblasts (Alsina *et al*., 2004) may be regulated differently from the mouse. The first signs of *cGata3* expression in the otic ganglion were detected at HH24, being associated specifically with the auditory neurons (III) as in the case of its mouse counterpart. The expression increased considerably at HH26 (III), coinciding similarly to the mouse (at E11.5–14.5) with the formation of postmitotic neurons, suggesting the conserved role in promoting the auditory neuron maturation and/or maintenance.

The expression of *Gata2* was specifically associated with the vestibular ganglion in both species. However, temporal differences in *Gata2* expression onset in developing ganglion were detected between these species. While the expression of *mGata2* appeared in the vestibular compartment of the formed ganglion at E11.5, *cGata2* was detectable already at HH21 in the antero-ventral otic epithelium where the neuroblasts migrate out and also in a subset of cells in the ganglion (I; III). In mouse embryos with conditionally inactivated *mGata2* the size of the vestibular ganglion appeared normal at E16.5 (unpublished data), indicating that Gata2 is not essential in the formation of the vestibular ganglion in the mouse.

In conclusion, the association of *Gata3* expression with auditory, *Gata2* with vestibular, and *Fgf10* with both neuron types was conserved between the mouse and the chicken. However, it remains to be seen if the temporal differences in the onset of the expression of the Gata genes between the mouse and the chicken indicate different roles for the *Gata* genes in these species, or alternatively, their roles are conserved, and the stages of neuroblast development vary temporally between the species.

CONCLUSIONS

1. Gata3 was essential for normal morphogenesis during otic epithelium invagination in mouse embryos. Without Gata3 the otic cup morphology was aberrant, and the formed vesicle was reduced or divided into two separate vesicles. The dissociated dorsal vesicle carried endolymphatic characteristics while the ventral one possessed features of the remaining main part of the otic epithelium.
2. The aberrant morphogenesis of early *Gata3–/–* mouse otic epithelium could result from altered epithelial properties, such as increased adhesion and reduced intraepithelial motility.
3. Gata3 was not required for major patterning of mouse otic epithelium as the dorso-ventral polarity was established normally, and no ectopic extension of dorsal or ventral genes was observed.
4. The expression of several dorsal genes was decreased in *Gata3–/–* mouse embryos, thus affecting the development of the dorsal structures, such as the semicircular and endolymphatic ducts. The decrease in expression could be due to the observed reduction of the contact between the neural tube and the otic epithelium, resulting in reduced access to neural tube derived signals.
5. Gata3 is not required for establishing the inner ear sensory primordia or for vestibular sensory differentiation. Instead, it is specifically important for the differentiation of auditory sensory epithelium in mouse embryos.
6. Gata3 was required to initiate *Fgf10* expression in mouse otic epithelium. The regulation was most likely direct since Gata3 could transactivate reporter gene expression from the *Fgf10* regulatory region that contained several conserved Gata3 binding sites. In addition, the early expression domains of *Gata3* and *Fgf10* overlapped considerably.
7. The early otic development occurred normally in *Gata2–/–* embryos; later the absence of Gata2 resulted in growth defects in the semicircular ducts. The expression domains of *Gata2* and *Gata3* were highly overlapping at early stages but diverged later coinciding with the appearance of *Gata2–/–* otic phenotype. Since *Gata3* expression was preserved in *Gata2–/–* ears, and these factors are known to bind similar DNA sites, the lack of early otic defects in *Gata2–/–* embryos could be due to redundant functions of Gata3.
8. Remarkable differences between *Gata3* expression patterns in the otic epithelia of the mouse and the chicken during invagination indicated evolutionary divergences in the molecular mechanisms involved in placode morphogenesis of birds and mammals.
9. Conservation of the *Fgf10* regulatory region responsible for Gata3 mediated transactivation together with the observed expression patterns of chicken *Gata3* and *Fgf10* indicate that Gata3 could regulate *Fgf10* also in chicken otic epithelium.

10. In both the mouse and the chicken, the expression domains of *Gata3* and *Gata2* were highly overlapping in early otic epithelium but became increasingly distinct later. While *Gata3* expression became restricted mostly to the auditory sensory epithelium and cochlear ganglion, *Gata2* expression was prominent in the vestibular non-sensory epithelium.

This study has provided new insights into the poorly understood early otic development. The described *Gata3–/–* otic phenotype is unique as it exhibits an ectopic morphological boundary in the medial wall of the otic cup epithelium, and disruption of the otic epithelium along that boundary is frequent. Interestingly, the ventral expression border of several dorsally expressed genes coincided with the ectopic boundary. In turn, the expression boundary formed at the site where the otic cup epithelium lost its close contact to the neural tube in wild type embryos. Thus this dorso-ventral boundary appears to be closely linked to dorsal gene expression and contact to the neural tube. A morphological boundary is revealed only in the absence of Gata3. These observations suggest that Gata3 regulates events that occur across the boundary. The data of the present study suggest that this event could be intra-epithelial cell migration or rearrangement as in *Drosophila* where a Gata factor controls a similar phenomenon. Understanding the exact meaning of this boundary will provide important knowledge about the mechanisms underlying early otic morphogenesis.

Semicircular ducts do not form in *Gata3–/–* embryos. Here the study showed the complex nature of this phenotype. Reduced contribution of cells from the ventral part of the otic cup epithelium towards the dorsal compartment, decreased expression of dorsal genes, and lack of Fgf10 may all contribute to the observed outcome even if the individual effects could be separated.

Haploinsufficiency of *Gata3* leads to deafness in mice and humans. The failure of sensory cell maintenance has been demonstrated in the mouse, but its possible reasons are unknown. The expression of *Gata3* in hair cells has been thought to influence cell-autonomously their maintenance (van der Wees *et al*., 2004). However the present study showed that *Gata3* is expressed also in the stria vascularis and in its chicken equivalent, the tegmentum vasculosum, the endolymph secreting and K^+ recycling tissue. For future therapy development, it is important to understand, whether Gata3 influences hair cell maintenance because its presence in hair cells or in endolymph secreting cells or both.

Understanding the molecular mechanisms responsible for cell cycle regulation and the differentiation of auditory hair cells are of great interest in the research on deafness therapies. The present study showed that Gata3 is specifically required for the generation of postmitotic hair and supporting cell progenitors in auditory prosensory epithelium. Further studies towards understanding the mechanisms through which Gata3 regulates auditory sensory differentiation will be of great importance.

Remarkable differences between *Gata3* expression in the mouse and the chicken during otic invagination strongly suggest non-conserved molecular control mechanisms over the earliest morphogenetic step when the otic placode forms a vesicle and reiterate that morphological conservation can be accomplished by harnessing diverged molecular control mechanisms. Accordingly, using both the mouse and the chicken as model organisms, it is possible to complement and enrich the knowledge about early otic development although the data cannot be extrapolated too straightforwardly.

SUMMARY IN ESTONIAN

Transkriptsioonifaktorid Gata3 ja Gata2 sisekõrva arengus

Sisekõrv on keerulise ehitusega membranoosne labürint selgroogsetes, mis tagab nii kuulmise kui ka organismi tasakaalu. Tasakaaluaparaat on erinevates selgroogsete klassides suhteliselt sarnane ja hõlmab sisekõrva keskmes paiknevat utriikulit, mis registreerib raskusjõudu ja joonkiirendust ning kolme selgmiselt paiknevat poolringkanalit, kus toimub asendi muutuste tajumine. Imetaja ja linnu tasakaaluaparaadi koostisesse kuulub veel utriikuliga sarnast funktsiooni täitev sakkul ehk kotike. Kontrastina tasakaaluaparaadile ei ole kuulmisaparaat evolutsiooniliselt kuigi konserveerunud. Erinevalt teistest selgroogsete klassidest on imetajatel ja lindudel helilainete vastuvõtmiseks kujunenud kohleaarjuha. Sisekõrva funktsionaalsuse tagavad piirkonniti koondunud mehhanoretseptoritena toimivad meelerakud – karvarakud. Eraldi väärib rõhutamist meelerakkude koepotentside erinevus: lindudel on sensoorne piirkond taastumisvõimeline, imetajatel aga mitte.

Sisekõrv ning sellele aferentset varustatust tagavad kõrvaganglioni neuronid arenevad ektodermaalsest kõrvaplakoodist, mis indutseeritakse pea katteektodermis paraksiaalse mesodermi ja tagaaju neuroepiteeli koostöö tulemusena. Kõrvaplakood sopistub sisse kõrvasüvendiks, mis sulgub kõrvavesiikuliks ja allub seejärel edasistele kiiretele morfogeneetilisele muutustele. Varajane kõrvaepiteel on tihedas ühenduses närvitoruga, millest lähtuvad signaalid on määrava tähtsusega kõrvaepiteeli mustri kujunemises, et tagada kiire ja suhteliselt sõltumatu hilisem areng.

Suunatud mutageneesi kasutamise abil on teadmised sisekõrva arengu molekulaarsest regulatsioonist viimasel aastakümnel küll oluliselt täienenud, kuid siiski esineb neis veel tõsiseid lünkasid. Seetõttu on aktuaalne jätkata uurimusi sisekõrva geneetilise regulatsiooni selgitamisel.

Morfoloogiliselt sarnaste struktuuride kujunemisel võivad osaleda erinevad molekulaarsed kontrollmehhanismid. Seega on huvitav võrrelda molekulaarseid erinevusi ja sarnasusi linnu ja imetaja sisekõrva arengus, kelle eelkäijad lahknesid ligikaudu 300 miljonit aastat tagasi. Lisaks on imetajate ja lindude kõrva arengu võrdlus oluline ka praktilisest küljest. Nagu eespool mainitud, suudab lindude sensoorne epiteel regenereeruda erinevalt imetajatest, kelle sensoorsed karvarakud ei taastu. Selle erinevuse molekulaarsete aluste mõistmine võib anda uusi lähtepunkte kurtuse ravile. Pealegi on lindude embrüod märksa kättesaadavamad ja kergemini manipuleeritavad *in vivo* uuringutes. Seega on vaja mõista kuivõrd võib kanda linnu kõrva arengu uurimisel tehtud avastusi üle imetajatele.

Käesoleva töö eesmärk oli tuua selgust kahe lähedase transkriptsioonifaktori, Gata3 ja Gata2 osalusse sisekõrva morfogeneesis ja sensoorses arengus ning

selgitada nende geenide ekspressioonide ja seega potentsiaalsete rollide konserveerumist imetajate ja lindude vahel. Varasemad tööd on näidanud, et transkriptsioonifaktor Gata3 on ekspresseerunud ajaliselt ja ruumiliselt selgelt piiritletuna ning tal on hiire sisekõrva kujunemises oluline osa (Karis *et al*., 2001). *Gata3–/–* hiire sisekõrva areng pidurdub oluliselt vesiikuli staadiumis, poolringkanalid ei moodustu ning kohlea jääb märkimisväärselt lühenenuks. Ent selle fenotüübi algupära ja molekulaarsed mehhanismid olid siiani jäänud teadmata, Gata3-le ei olnud sisekõrvas teada ka ühtegi märklaudgeeni. Lisaks põhjustab *Gata3* heterosügootsus kurtust nii hiirel kui inimesel. Hiirel on näidatud, et kuulmise kadu on tingitud karvarakkude hävimisest. Gata2 ekpressioon ja funktsioon olid sisekõrva arengus seni uurimata.

Uurimuse tulemusena selgus, et *Gata3* ekspressiooni puudumine põhjustab varajasi häired kõrvaepiteeli morfogeneesis. Kõrvaplakood kujunes normaalselt, kuid kõrvasüvendi morfoloogia oli vigane ning selle sulgumisel jagunes kõrvaepiteel sageli kaheks. Rakkude kaardistamise abil on näidatud, et kõrvaplakoodi morfogeneesi vältel toimuvad laialdased epiteelisisesed rakkude liikumised, mille tagajärjel lisandub kõrvaepiteeli kõhtmiste rakkude seast oluline osa selgmisse domeeni (Brigande *et al*., 2000a). Laiaskaalaline ekspressioonianalüüs viitas, et ebanormaalse kõrva morfoloogia tekkimise taga *Gata3–/–* embrüotes on tõenäoliselt muutused rakkude adhesioonis ja sellega kaasnevalt vähenenud epiteelisiseses liikuvuses. Seega on suure tõenäosusega häiritud aktiivselt jagunevate kõhtmise domeeni rakkude lisandumine tulevasse endolümfaatilisse domeeni. Kirjeldatud olukord viib sageli endolümfaatilise domeeni eraldumiseni.

Töö tulemused näitasid, et kõrvavesiikuli selgmises piirkonnas ekspresseerunud geenide ekspressioonidomeenid olid oluliselt kitsenenud ning samuti ekspressiooni tasemed alanenud. Teadaolevalt on neuraalkoest lähtuvad signaalid määrava tähtsusega külgneva kõrvaepiteeli musterdumises. Kuna vigase morfogeneesi tulemusena jäi kõrvaepiteeli ja närvitoru vaheline kontakt puudulikuks, oli kõrvaepiteeli selgmiste geenide ekspressiooni vähenemine suure tõenäosusega tingitud närvikoest tulenevate signaalide väiksemast kättesaadavusest. Varajase kõrvaepiteeli rakkude liikuvuse alanemine koos selgmiste geenide ekspressiooni vähenemisega on tõenäoliselt peamised poolringkanalite puudumise põhjused *Gata3* mutantsetes embrüotes.

Uuringutest selgus, et *Fgf10* on hiire sisekõrvas Gata3 märklaudgeen. Transaktivatsiooniuuringud rakukultuuris näitasid, et Gata3 võib otseselt reguleerida *Fgf10* ekspressiooni. Fgf10 puudumine *Gata3* mutantses sisekõrvas lisab oma osa poolringkanalite mutantsesse fenotüüpi, sest *Fgf10* mutantidel on poolringkanalite moodustumine tõsiselt häiritud.

Gata3 puudulikus kõrvaepiteelis toimus sensoorne määratlemine, ent hilisemas diferentseeumises oli Gata3 spetsiifiliselt vajalik auditiivse sensoorse epiteeli diferentseerumiseks. Antud tulemuse edasine uurimine on oluline kuulmisepiteeli diferentseerumise molekulaarsete aluste mõistmiseks, mis võib omakorda aidata kujundada edasisi kurtuse ravivõimalusi.

Kontrastina *Gata3* mutandile arenes *Gata2–/–* kõrvavesiikul nähtavate puudusteta. *Gata2* mutantsetel hiireembrüotel avaldus kõrvafenotüüp hilisemates staadiumides kasvupuudujäägina poolringkanalites. Hiline kõrvafenotüübi ilmnemine *Gata2* mutantides on tõenäoliselt põhjustatud Gata2 funktsioonide kattuvusest Gata3 omadega. Nimelt oli *Gata3* normaalselt ekspresseerunud arenevas *Gata2–/–* sisekõrvas ning mõlema *Gata* geeni ekspressioonidomeenid kattusid oluliselt varajases kõrvavesiikulis. Pealegi toimus *Gata3* vestibulaarse ekspressiooni alla reguleerimine samaaegselt kõrvafenotüübi ilmnemisega *Gata2* mutandis.

Gata3 ja *Gata2* ekspressioonimustrite võrdlev analüüs hiires ja kanas näitas olulist konserveerumist hilisemates staadiumides, kus *Gata3* oli tugevalt ekspresseerunud kohlea sensoorses piirkonnas ning *Gata2* tasakaaluaparaadi mittesensoorses epiteelis. Need tulemused viitavad konserveerunud rollidele hilisemates etappides. Oluline erinevus avaldus aga *Gata3* ekspressioonis hiire ja kana kõrvaplakoodi sissesopistumise käigus: kui hiire *Gata3* ekspressioon oli täheldatav üle kogu kõrvaepiteeli tugevaima mediaalse domeeniga (närvitoru poolselt), siis kana Gata3 oli ekspresseerunud vaid kõrvasüvendi külgmises servas, puududes täielikult mediaalsest domeenist. Kirjeldatud erinevus viitab lindude ja imetajate kõrvaplakoodi morfogeneesi kontrollivate molekulaarsete mehhanismide evolutsioonilisele lahknemisele. Seega täiendavad hiire ja kana kõrva varajase arengu uurimisest saadud teadmised teineteist kõrvaplakoodi morfogeneesi regulatsiooni mõistmisel, kuid saadud tulemusi ei või üheselt üle kanda ühelt organismilt teisele.

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