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Research paper

Defects in sensory organ morphogenesis and generation of cochlear hair cells in *Gata3*-deficient mouse embryos

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ABSTRACT

The development of the inner ear sensory epithelia involves a complex network of transcription factors and signaling pathways and the whole process is not yet entirely understood. GATA3 is a DNA-binding factor that is necessary for otic morphogenesis and without GATA3 variable defects have been observed already at early stages in mouse embryos. In the less severe phenotypes, one small oval shaped vesicle is formed whereas in the more severe cases, the otic epithelium becomes disrupted and the endolymphatic domain becomes separated from the rest of the otic epithelium. Despite these defects, the early sensory fate specification occurs in *Gata3*−/− otic epithelium. However, due to the early lethality of *Gata3*-deficient embryos, the later morphogenesis and sensory development have remained unclear. To gain information of these later processes we produced drug-rescued *Gata3*−/− embryos that survived up to late gestation. In these older *Gata3*−/− embryos, a similar variability was observed as earlier. In the more severely affected ears, the development of the separate endolymphatic domain arrested completely whereas the remaining vesicle formed an empty cavity with variable forms, but without any distinguishable otic compartments or morphologically distinct sensory organs. However, the dorsal part of this vesicle was able to adopt a sensory fate and to produce some hair cells. In the less severe cases of *Gata3* −/− ears, distinct utricular, saccular and cochlear compartments were present and hair cells could be detected in the vestibular sensory epithelia. Although clear cristae and maculae formed, the morphology and size of these sensory areas were abnormal and they remained often un-separated. In contrast to the vestibule, the cochlear sensory compartment remained more immature and no hair or supporting cells could be detected. Our results suggest that GATA3 is critical for normal vestibular and cochlear morphogenesis and that it is especially important for cochlear sensory differentiation.

1. Introduction

Inner ear is a membranous labyrinth that comprises three types of sensory epithelia in mammals. The vestibular compartment includes cristae in one end of each three semicircular ducts and maculae in the utricle and saccule. The vestibular compartment collectively detects linear and angular head movements and gravity to maintain balance. In the ventral part, the cochlear duct houses the organ of Corti for sound wave detection (as reviewed in Noramly and Grainger, 2002; Chatterjee et al., 2010). The different sensory epithelia consist of mechanosensory hair cells and different sets of supporting cells which are thought to develop from a common prosensory region in the ventromedial wall of the early otic vesicle (as reviewed in Bryant et al., 2002; Fekete and Wu, 2002). The development of the sensory epithelia involves several partially overlapping steps including proliferation of the multipotent progenitor cells, prosensory cell specification, subdivision of the sensory competent epithelium to form the separate organs, cell cycle exit, cell fate determination, differentiation, maturation and survival (as reviewed in Kelley, 2006; Puligilla and Kelley, 2009; Kamaid et al., 2010).

Several signaling pathways and transcription factors have been implicated in the sensory development of the vertebrate inner ear. Bone morphogenetic protein (BMP) and NOTCH signaling as well as on the transcription factor SOX2 that belongs to the SRY (sex

Abbreviations: BrdU, bromodeoxyuridine; GER, greater epithelial ridge; LER, lesser epithelial ridge; nls, nuclear localization signal; ZNPC, zone of non-proliferating cells.

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determining region Y) -box family have been implicated in prosensory specification (Dabdoub et al., 2008; Hartman et al., 2010; Ohyama et al., 2010). In addition, also Fibroblast growth factor (FGF) signaling and the transcription factors Eyes absent homolog 1 (EYA1) are thought to participate in the regulation of prosensory fate (Hayashi et al., 2008; Zou et al., 2008). The timing of prosensory fate establishment is still unclear, but the expression of NOTCH ligand *Jagged1* (*Jag1*), *Bmp4* and the NOTCH pathway modulator *Lunatic fringe* (*Lfng)* in the prosensory epithelia at E9.5–10.5 in mouse embryos suggests that the identity has been established by then (Morsli et al., 1998; Morrison et al., 1999). Prosensory specification is followed by cell cycle withdrawal and Cyclin dependent kinase inhibitor 1B (CDKN1B, also called $p27^{Kip1}$) is required for the correct timing of terminal mitoses in the cochlea (Chen and Segil, 1999; Lee et al., 2006). Subsequently, the proneural factor ATOH1 (also called MATH1) is required for hair cell differentiation together with the NOTCH pathway whereas FGF8 and FGF receptor 3 (FGFR3) induce some of the supporting cells to differentiate (Bermingham et al., 1999; Hayashi et al., 2007; Jacques et al., 2007; Pan et al., 2010). Furthermore, the terminal differentiation and/or survival of hair cells is dependent on a POU domain factor, POU4F3, and inactivation of mouse *Pou4f3* causes a progressive loss of both vestibular and cochlear hair cells leading to a secondary defect in supporting cells (Erkman et al., 1996; Xiang et al., 1997, 1998).

The GATA-sequence binding transcription factor family consisting of GATA1–6 has numerous specific roles during vertebrate development (as reviewed in Patient and McGhee, 2002). Two family members, *Gata2* and *Gata3,* are expressed in a highly conserved manner in chicken and mouse inner ear during development. Although their expression overlaps considerably in the early otic vesicle, it becomes increasingly distinct at later stages so that *Gata2* is confined to the non-sensory parts and *Gata3* in the sensory epithelia especially in the cochlea (Lilleväli et al., 2004, 2007). The inactivation of *Gata2* in mouse leads to relatively late developmental defects observed around E15.5 in the growth of the semicircular ducts and in the clearance of the mesenchymal cells from the perilymphatic space between the otic epithelium and the bony capsule (Haugas et al., 2010). Instead, *Gata3*-deficient mouse embryos suffer from very early morphogenetic problems detected already at E8.5–9.5 including perturbation in otic placode invagination and disruption of the otic epithelium into two parts forming a small dorsal and a larger ventral vesicle with corresponding gene expression identities (Lilleväli et al., 2006). The phenotype is however variable so that in half of the ears, less severe defects are detected and the otic vesicle stays intact, but is nevertheless unable to undergo normal morphogenesis and growth (Karis et al., 2001; Lilleväli et al., 2006). Despite these morphological defects, the initiation of the prosensory development appears to occur normally in *Gata3*−/− otic vesicles as judged by the expression of *Lfng* and *Bmp4* in the early sensory competent domains (Lilleväli et al., 2006). Whether the sensory development proceeds further has remained unclear due to the lethality of *Gata3*−/− embryos usually around E11.5 (Pandolfi et al., 1995; Karis et al., 2001). However, the persisting and conserved expression of *Gata3* in the developing and adult organ of Corti (Lilleväli et al., 2004; Van der Wees et al., 2004) suggest important roles in the formation and/or maintenance of the cochlear sensory epithelium. In fact, adult *Gata3*+/− mice suffer from degeneration of cochlear hair and supporting cells and progressive hearing loss (Van der Wees et al., 2004). In addition, haploinsufficiency of GATA3 in humans results in the HDR syndrome defined by hypoparathyroidism, sensorineural deafness and renal dysplasia (Van Esch et al., 2000; Nesbit et al., 2004).

To get novel insights into the potential roles of GATA3 in inner ear sensory development we used a pharmacological-rescue regime that supports the development of tissues depending on adrenergic innervation and enables the *Gata3*−/− embryos to survive beyond E11.5 (Lim et al., 2000; Kaufman et al., 2003). We show that without GATA3 the sensory organ development is severely impaired in both the vestibule and the cochlea. While the vestibular sensory epithelium succeeded however in producing a limited number of *Atoh1* and *Pou4f3* expressing hair cells, the cochlear sensory epithelium remained more immature and no evidence for sensory differentiation could be detected.

2. Material and methods

2.1. Mouse breeding and genotyping

The generation and genotyping of *Gata3*−/− (*Gata3*$^{nlslacZ/nlslacZ}$) mice has been described previously (Hendriks et al., 1999; Lilleväli et al., 2004). The mice were kept in a C57bl/6 background and embryos were taken from timed matings and the day on which a vaginal plug was detected was assigned as E0.5. To obtain E12.5–17.5 *Gata3*−/− embryos, pregnant females were treated with a drug-rescue regime (Kaufman et al., 2003) giving them 125 μg/ml L-phenylephrine, 125 μg/ml isoproterenol and 2 mg/ml ascorbic acid (all from Sigma) daily in fresh water starting from E7.5. Wild type or *Gata3*+/− embryos from the same litters as the rescued *Gata3*−/− embryos were used as controls. No phenotypic differences were observed between the different control embryos. At least 3 ears/genotype/phenotype/stage were used for each analysis. All mouse work was performed according to the ethical regulations of the University of Tartu.

2.2. RNA in situ hybridization, X-gal staining, immunohistochemistry and detection of cell proliferation

The radioactive in situ hybridizations were performed on 10-μm serial paraffin sections as before (Salminen et al., 2000) and the images were treated as in Lilleväli et al. (2004) to produce the red pseudocolor to indicate gene expression. The antisense and sense probes were prepared as described in Salminen et al. (2000). No specific signals were detected with any of the sense probes. The paraffin sections were counterstained with hematoxylin.

X-gal staining in whole embryos to visualize the *Gata3* driven β-galactosidase (β-gal) activity was performed as before (Salminen et al., 1998). The 4% paraformaldehyde fixed E10.5 embryos were then embedded in gelatin and 25-μm sections were cut with Vibratome. The E12.5 embryos were embedded in paraffin and 10-μm sections were cut with Microtome, the sections were deparafinated and counterstained with eosin before photography.

For immunohistochemistry, serial 10-μm paraffin embedded sections were deparafinated and treated with Antigen unmasking solution (Vector). Sections were blocked for 30 min at room temperature with 10% goat serum and 0.3% triton X-100 in PBS followed by incubation with primary antibody overnight at +4 °C. Antibody dilutions: calretinin 1:200 (Chemicon), SOX2 1:400 (Chemicon), GATA2 1:200 (Santa Cruz), GATA3 1:200 (Santa Cruz), PROX1 1:100 (Acris Antibodies). Secondary antibodies were Alexa Fluor 1:500 (Molecular Probes).

For cell proliferation assays, pregnant females were injected intraperitoneally with BrdU-solution 1.5–2 h prior sacrifice and detection of proliferating cells in E14.5 embryos was performed as before (Salminen et al., 2000). The paraffin embedded sections were analyzed with anti-BrdU-antibody (DAKO) as before (Salminen et al., 2000).

All antibody-stained sections were mounted in Vectashield (Vector) and photographed with Olympus DP70 CCD-camera attached to Olympus AX70 microscope. The plane of all paraffin sections is indicated in Fig. 1Q–S.