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Nkx5-1 controls semicircular canal formation in the mouse inner ear

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SUMMARY

The inner ear develops from the otic vesicle, a one-cell-thick epithelium, which eventually transforms into highly complex structures including the sensory organs for balance (vestibulum) and hearing (cochlea). Several mouse inner ear mutations with hearing and balance defects have been described but for most the underlying genes have not been identified, for example, the genes controlling the development of the vestibular organs. Here, we report the inactivation of the homeobox gene, *Nkx5-1*, by homologous recombination in mice. This gene is expressed in vestibular structures throughout inner ear development. Mice carrying the Nkx5-1 null mutation exhibit behavioural abnormalities that resemble the typical hyperactivity and circling movements of the shaker/waltzer type mutants. The balance defect correlates with severe malformations of the vestibular organ in Nkx5-1$^{-/-}$ mutants, which fail to develop the semicircular canals. Nkx5-1 is the first ear-specific molecule identified to play a crucial role in the formation of the mammalian vestibular system.

Key words: *Nkx5* genes, Inner ear, Vestibular development, Mouse, Otic vesicle, Cochlea

INTRODUCTION

The inner ear anlagen arise at both sides of the hindbrain at the level of the prospective rhombomeres 5 and 6 as single-cell-layered epithelium of the otic placodes, which subsequently invaginate and eventually close into the otic vesicles (otocysts). The future fate of individual structures of the inner ear is believed to be defined very early at the placode and vesicle stages, although few functional data exist to support this assumption (Anniko and Wikström, 1984; Fekete, 1996). After closure of the vesicle, remarkable morphogenetic movements lead to the establishment of the organs for balance and hearing. The adult inner ear can be divided into the auditory apparatus, including the organ of Corti within the coiled cochlea, which is responsible for sound perception, and the vestibular apparatus, which is responsible for the sense of balance. The vestibular part consists of two principal sets of structures, the otolith organs (sacculus and utriculus) and three semicircular canals. The semicircular canals build the bony scaffold for the epithelial semicircular ducts. The semicircular ducts are arranged in three dimensions more or less perpendicularly to each other. They originate from bilayered outpocketings emerging at E11.5 (embryonic day 11.5) in a timely ordered fashion: the anterior canal is formed first with the posterior lagging slightly behind. The lateral semicircular canal develops from a separate rudiment about a half day later, at E12.0 (Sher, 1971; Martin and Swanson, 1993). The lateral wall of each epithelial outpocketing delaminates from the underlying mesenchyme and grows towards the corresponding medial wall forming the fusion plate (Martin and Swanson, 1993; Fekete et al., 1997). Subsequently, the fusion plate has to disappear in order to form the closed, tubular form of the canal. Elimination of cells within the fusion plate is a critical step in canal morphogenesis and the underlying mechanisms are still unclear. In the mouse, the recruitment of the fusion plate cells back into the canal epithelium was proposed as a principal mechanism of canal formation (Martin and Swanson, 1993) and the role of cell death was demonstrated elegantly in the chick by Fekete et al. (1997). After initial formation of the canal tube, each semicircular canal widens at its base into the ampulla. In the ampullae, sensory receptor cells are located within cristae ampullaris. As a consequence of their arrangement in three-dimensional space, the cristae detect angular acceleration of the head in any of these three directions, whereas the maculae of the otolith organs are responsible for linear acceleration (Kelly, 1991).

Although specific expression patterns in otic vesicles have been described for several genes (reviewed in Fekete, 1996), functional importance in early morphogenetic processes has only been demonstrated for the *Pax2* gene within the cochlea. This gene is necessary for the outgrowth of the future cochlea from the ventromedial part of the otocyst (Torres et al., 1996). Furthermore, inactivation of genes that are primarily responsible for the hindbrain organization, in some cases, resulted in inner ear defects (Mansour et al., 1993; Cordes and Barsh, 1994; Mark et al., 1993). Another group of identified single gene defects comprises molecules expressed in specialized cell types, such as the hair cells during later stages (Gibson et al., 1995; Ernfors et al., 1995; Erkman et al., 1996; Vetter et al., 1996). We have previously identified two *NK*-related homeobox genes, *Nkx5-1* and *Nkx5-2*, which showed specific expression during inner ear development and later in

distinct structures of the peripheral and central nervous system (Bober et al., 1994a). *Nkx5-1* begins to be expressed in the otic placode at E8.5 and exhibits dynamic changes of the expression pattern during otic vesicle formation. *Nkx5-2* transcripts begin to accumulate in the same structures almost 5 days later at E13.5 of mouse development (Rinkwitz-Brandt et al., 1995, 1996). *Nkx5-1* is first expressed in the rostral part of the otic placode and relocates during otic vesicle formation from an originally medial domain to the dorsolateral wall (Rinkwitz-Brandt et al., 1996; see also Fig. 4A). This region later gives rise to the vestibular apparatus of the inner ear (Li et al., 1978). The unique and interesting *Nkx5-1* expression pattern in the otocyst reflecting the complex morphogenetic events occurring in the seemingly homogenous epithelial sac prompted us to investigate the role of the *Nkx5-1* gene by loss-of-function mutation using homologous recombination in ES cells.

MATERIALS AND METHODS

Construction of the targeting vector

Nkx5-1 gene was isolated from a genomic phage library from J1-ES cell DNA (a kind gift of R. Jaenish) by screening with a mouse *Nkx5-1* cDNA (Bober et al., 1994a). Several overlapping phage clones were isolated which covered about 40 kb of the locus. For the construction of the targeting vector, a 9.5 kb partial *Bam*HI fragment containing the entire coding region and the 5′ and 3′ flanking sequences was used. This fragment was cut with *Xho*I and the resulting 5′ *Bam*HI/*Xho*I and 3′ *Xho*I/*Sal*I (linker enzyme in this particular phage clone) fragments cloned into the suitable sites of the pPNT transfer vector.

Generation of Nkx5-1 knockout mutants

Embryonic stem cell line J1 was grown on embryonic feeder cells as described (Braun et al., 1992). Electroporation and selection of ES cells were all done as described previously (Braun et al., 1992). A 10 kb mutant *Eco*RI fragment hybridizing to the probe 1 (see Fig. 1A) was detected in 62 of the total of 280 cell clones tested. Three randomly chosen recombinant ES cell clones were injected into C57/BL6 mouse blastocysts. Two clones produced chimeric animals and one of the chimeras resulted in the germline transmission. This chimeric animal was used to establish inbred and C57/BL6 hybrid mouse strains.

Hearing tests

Hearing was assessed using behavioural tests, specifically the Preyer reflex (Steel and Harvey, 1992). A brief (1-3 seconds) 20 kHz soundburst at the intensity of 90-100 dB was generating by a function generator (PM 5108L, Philips). A loudspeaker (monitor speaker model MS 60S, Yamaha) was placed about 30 cm directly above the mouse. A flick backwards of the pinna upon hearing the sound was counted as a positive Preyer response.

Whole-mount in situ hybridisation

Whole-mount in situ hybridisation experiments were performed as previously described (Bober et al., 1994b). Antisense RNA probes using for detection of *Nkx5-1* and *Pax2* transcripts were described in Rinkwitz-Brandt et al. (1995). For generation of the *Msx1* probe, a 1.2 kb cDNA fragment was used (Hill et al., 1989).

Embryos were embedded in a mixture of 0.5% gelatine, 30% albumin, 20% sucrose and 2% glutaraldehyde and sectioned at 30 μm using the Leica Jung Autocut vibratome. Sections were photographed under a Zeiss Axiovert microscope with Nomarski optics. Whole-mount embryos were photographed under a Wild M10 stereomicroscope.

Isolation of the inner ears

Inner ears were isolated from 3-week-old Nkx5-1$^{-/-}$ and wild-type mice. The attached tissue was dissected off using thin forceps and the inner ears were fixed in 4% paraformaldehyde, washed, dehydrated and cleared in methyl salicylate as described (Martin and Swanson, 1993).

In situ hybridisation on paraffin sections

In situ hybridisations were performed on 10 μm thick paraffin tissue sections as described previously (Bober et al., 1994a). The *Nkx5-1* and *Nkx5-2* antisense RNA probes were prepared also as described (Bober et al., 1994a; Rinkwitz-Brandt et al., 1995).

RESULTS

The *Nkx5-1* targeting vector was constructed to delete exon 1 and parts of exon 2 including the N-terminal one third of the homeobox DNA-binding domain. The resulting gene mutation prevents the production of a functional protein due to the large deletion (Fig. 1). In fact, no *Nkx5-1* transcripts were detected by in situ hybridisation in homozygous mutant mice, while heterozygotes accumulated apparently lower levels of transcripts than wild-type animals (Fig. 1C). Homozygous Nkx5-1 mutants were born alive at Mendelian ratios and revealed no gross morphological abnormalities except for a slight but significant growth retardation (not shown). Approximately 3 weeks after birth, the homozygous mutants started to show abnormal hyperactivity and circling movements which were suggestive of balance defects (Deol, 1983; Steel, 1991). In order to investigate whether the balance defects were caused by malformation of the vestibular organ inner ears of 3-week-old mice were isolated for morphological examination. While the inner ears of heterozygous Nkx5-1 mutants were indistinguishable from wild type, ears of homozygous mutant mice lacked major parts of the vestibular apparatus but showed no apparent alterations of the cochlea. Fig. 2 shows typical vestibular malformations of a Nkx5-1 mutant inner ear. In this Nkx5-1$^{-/-}$ mouse, the posterior and lateral semicircular canals were completely missing whereas remnants of the anterior semicircular canal were still present. The absence of the lateral canal was confirmed by transversal and sagittal sections through inner ears of several 3-week-old Nkx5-1$^{-/-}$ mutant mice (Fig. 3). The severity of dysgenesis, however, was variable for the posterior and anterior semicircular canals among individual mutant animals (n=6; penetrance) and between both ears within individuals (expressivity). We could not observe any apparent relationship between the penetrance of the phenotype and the genetic background but the relatively small number of individuals tested so far does not allow a final conclusion. Defects of the posterior canal ranged from entirely absent (Fig. 3B,D) to partly present (Fig. 3G). In no case was the posterior canal fully developed. The anterior semicircular canal had always evaginated but was significantly reduced in size in most mutant animals. These anatomical defects became apparent because the cartilaginous capsules that normally surround the semicircular ducts and their ampullae were not formed correctly (Fig. 3A-D,F,G). The remnants of the duct epithelium could often be recognized in the central cystic space (not shown). As described by Martin and Swanson (1993), the first critical steps during semicircular canal morphogenesis

take place between E11.5 and E13.5 and include the formation and fusion of canal plates followed by disappearance of the canal plate cells. The histological examination of sections of E12.5 embryos revealed the normal formation of the canal plates. However, more elaborate morphological analysis including EM studies will be necessary to assess more exactly the time point and origin of the defect.

Interestingly, the sensory epithelium of the cristae ampullaris was present in mutants except for the crista of the lateral semicircular duct which never formed. Similarly, the maculae of the utriculus and sacculus were also present (Fig. 3G and not shown). This is consistent with the fact that sensory epithelium does not express *Nkx5-1* (Rinkwitz-Brandt et al., 1996). In situ hybridisations on inner ear sections of E14.5 embryos with *Nkx5-2* probe revealed that this closely related gene was expressed normally in mutants and that epithelial cells that normally give rise to the semicircular ducts were present but built a cystic cavity which lacked the surrounding cartilage normally forming the canals (Fig. 3I,J). Whether these *Nkx5-2*-positive cells failed to evaginate or induce the surrounding cartilage framework in the mutant ears remains to be established.

In contrast to the dramatic malformations of the vestibular system, the morphology of the cochlea appeared essentially unaffected in mutants indicating that Nkx5-1 plays no significant role in this part of the inner ear (Fig. 3F,G). The endolymphatic duct also seemed to develop normally as revealed by sections of E14.5 mutant inner ear (Fig. 3J). Consistent with these observations no hearing problems were observed in young Nkx5-1 mutant animals (see Table 1). All four Nkx5-1$^{-/-}$ mice that have been analysed for the Preyer reflex (see Materials and Methods) showed clear responses comparable to those elicited by wild-type and heterozygous Nkx5-1, kreisler and homozygous fidget mice. Interestingly, one of the Nkx5-1 mutant mice extended the Preyer reflex into the startle response (the whole body jumped). Such startle response has not been observed in any of the homozygous fidget mice. The homozygous kreisler mice, which were tested as non-hearing controls, showed no response to the applied sound stimulus. The age of the tested Nkx5-1 homozygotes ranged from 4 to 6 months. Thus, it remains open whether age-dependent hearing loss develops in older individuals. In general, the isolated, strictly vestibular phenotype of Nkx5-1$^{-/-}$ mice suggests that individual parts of the inner ear develop independently. In line with this hypothesis, expression of the *Nkx5-1* becomes confined to the dorsolateral wall of the otocyst shortly after its closure (Fig. 4A). Fate map experiments demonstrated that this region gives rise to vestibular structures, although a more refined analysis is certainly required to map more precisely the distribution of individual cell lineages during inner ear morphogenesis (Li et al., 1978; Fekete, 1996).

To evaluate possible interdependent regulatory circuits between different parts of the developing inner ear, mutant and wild-type embryos were probed with markers for distinct presumptive inner ear organs. Pax2, as already mentioned, is crucial for the cochlea development (Torres et al., 1996). Its medioventral expression in the otic vesicle correlated well with the cochlear phenotype and fate maps. Fig. 4A demonstrates that *Nkx5-1* expression changed during placode and early vesicle stages. Its initial expression domain moved away from the position adjacent to the neural tube (see 12- and 26-somite stages in Fig. 4A) and became confined to the lateral wall of the closed otocyst (35-somite stage). *Pax2* was expressed in a complementary fashion to *Nkx5-1* in wild-type otic vesicles (compare Fig. 4A and B) and remained apparently unchanged in mutant otocysts (Fig. 4B). These complementary expression domains of *Pax2* and *Nkx5-1* genes in the wild type and unchanged *Pax2* expression in the Nkx5-1 null mutant indicate that similar functional complementarity as well as early segregation of the functional domains exist already in vesicle stages during the inner ear development. The second gene that has been analysed, *Msx1*, is normally expressed overlappingly to *Nkx5-1* in the lateral wall of the otocyst at 35-somite stage (compare Fig. 4A and B). Because *Msx1* is expressed in the same area but is activated later than *Nkx5-1* (Bober, unpublished observations), it constituted a possible downstream target of Nkx5-1. However, we could not detect any changes of *Msx1* expression in the mutant ear (Fig. 4B). Interestingly, later during inner ear organogenesis, *Msx1* gene is expressed in cristae ampullaris and shows no overlap with *Nkx5-1* gene expression (see Discussion).

Table 1. Results of the behavioural hearing tests

Genotype	Number of individuals tested	Preyer reflex positive
Wild type	4	4
Nkx5-1$^{+/-}$	3	3
Nkx5-1$^{-/-}$	4	4
fidget$^{-/-}$	2	2
kreisler$^{+/-}$	2	2
kreisler$^{-/-}$	2	0

Wild type, Nkx5-1 heterozygous and homozygous mutant mice along with control mice bearing inner ear mutations which can (fidget) or cannot (kreisler) hear were tested for the presence of Preyer reflex (see Material and Methods).

DISCUSSION

Inactivation of the *Nkx5-1* homeobox gene in the mouse leads to severe malformations of the semicircular canals of the inner ear. The complete lack of the lateral semicircular canal and lack or large reduction of the posterior and anterior canals coincide with the abnormal hyperactive and circling behaviour observed in Nkx5-1$^{-/-}$ mice. A similar behaviour has been described in several shaker/waltzer type mouse mutants. In some of these mutants, vestibular dysfunctions are caused by defects of CNS structures at different levels involved in conduction and processing of peripheral signals to vestibular centres. In another group of shaker/waltzer mutants, the circling behaviour appears to be due to defects within the primary sensory organ (Steel, 1991). The malformations observed within the vestibular apparatus of the Nkx5-1 null mutants strongly suggest that they belong to the latter category. Although the *Nkx5-1* gene appears not to be expressed in CNS structures that are involved in vestibular sensory pathways (Rinkwitz-Brandt et al., 1995), the involvement of the CNS components in the manifestation of the phenotype cannot be completely ruled out. The second problem not definitively resolved by this report is the role of Nkx5-1 in the development of the stria vascularis. Beginning at E17.5, *Nkx5-1* and the closely related *Nkx5-2* gene are both expressed in this cochlear

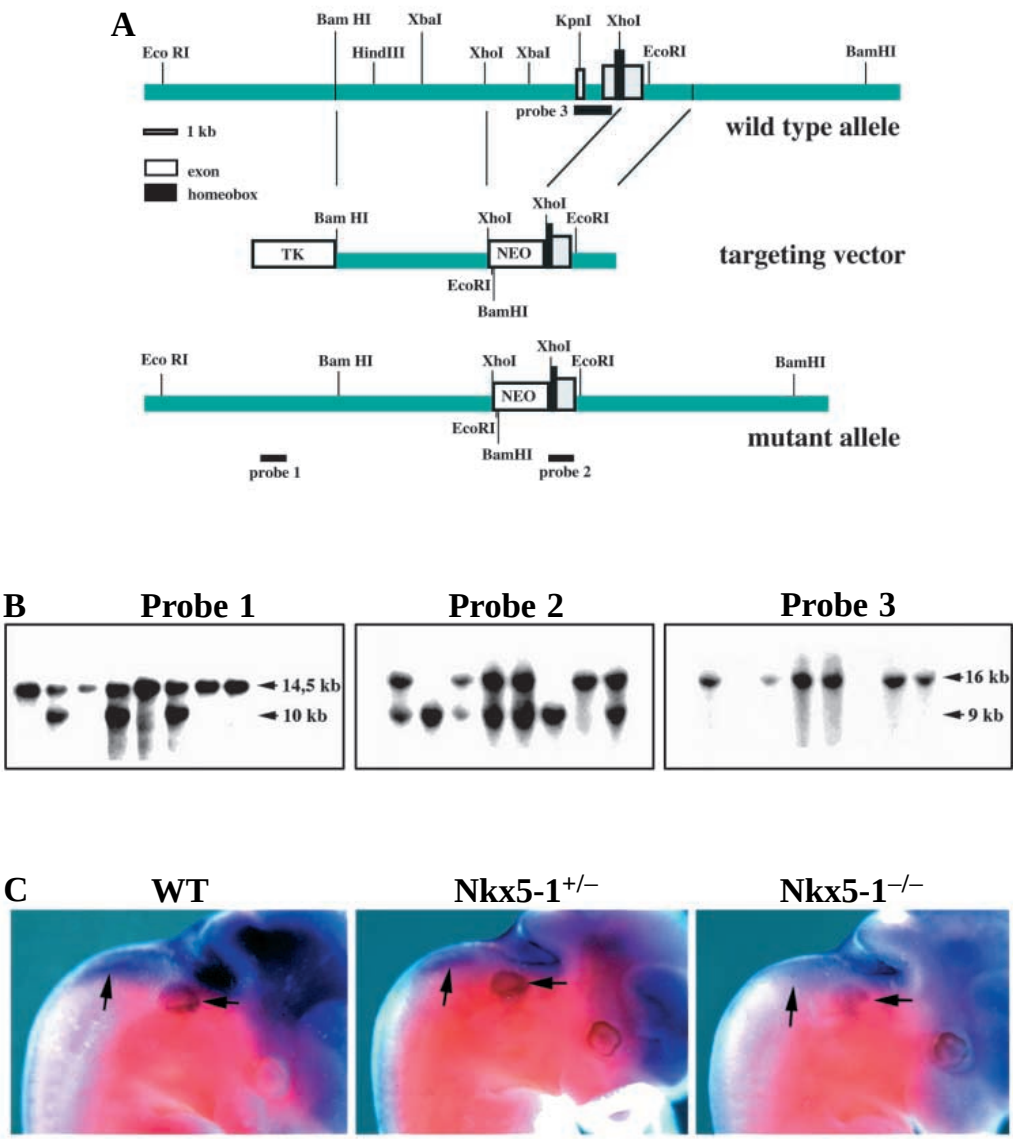

Fig. 1. Inactivation of the mouse *Nkx5-1* gene by homologous recombination. (A) The targeting vector contains a 4.5 kb *Bam*HI/*Xho*I 5′ genomic fragment inserted between the *TK* and *neo* gene cassettes in the opposite transcriptional orientation. A 2 kb *Nkx5-1* genomic fragment beginning at the *Xho*I site within the homeobox was cloned downstream of the *neo* gene. The mutant allele results in the replacement of a 4 kb *Xho*I fragment by the *neo* gene cassette. Probes used for Southern blot analysis are indicated by filled bars in A. (B) DNA isolated from ES cells was digested with *Eco*RI and hybridised with probe 1. The smaller 10 kb *Eco*RI fragment due to an additional *Eco*RI site within the *neo* gene indicates successful homologous recombination. DNA isolated from mice tails was digested with *Bam*HI and hybridised with probes 2 and 3. 16 kb and 9 kb *Bam*HI fragments indicate the wild-type and mutant alleles, respectively. Probe 3 detects only the wild-type *Bam*HI fragment. (C) Wild-type, heterozygous and homozygous E11.5 embryos were subjected to whole-mount in situ hybridisation with *Nkx5-1* antisense RNA probe. Arrows mark a hindbrain domain and otic vesicle, which both hybridise in a wild type. The hybridisation signals are significantly weaker in the heterozygotes and completely absent in homozygous Nkx5-1 mutant mice.

structure (Rinkwitz-Brandt et al., 1996). Stria vascularis is necessary for the establishment and maintenance of the endocochlear potential in the endolymph and its dysfunction results in hearing deficiency (Steel, 1995). As testing of the Preyer reflex in Nkx5-1 homozygotes does not allow to detect minor hearing defects, the more detailed assessment of the hearing ability in Nkx5-1 mutants as well as testing of older animals for possible late-onset hearing deficiency will clarify whether Nkx5-1 protein is involved in controlling the strial function. However, the simultaneously expressed *Nkx5-2* gene

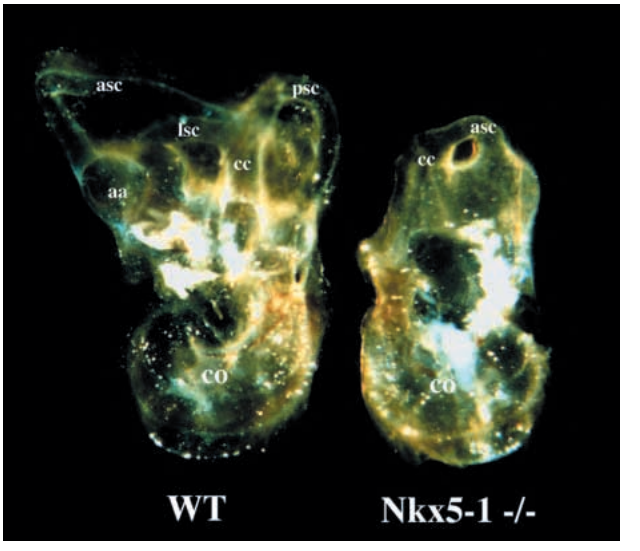

Fig. 2. The vestibular apparatus is severely malformed in Nkx5-1$^{-/-}$ mice. Isolated and methyl salicylate cleared inner ears of 3-week-old wild-type (WT) and Nkx5-1 null mutant mice are shown. There is no formation of the lateral and posterior semicircular canals in the mutant ear. Abbreviations: aa, ampulla of the anterior semicircular canal; asc, anterior semicircular canal; cc, crus commune; co, cochlea; lsc, lateral semicircular canal; psc, posterior semicircular canal.

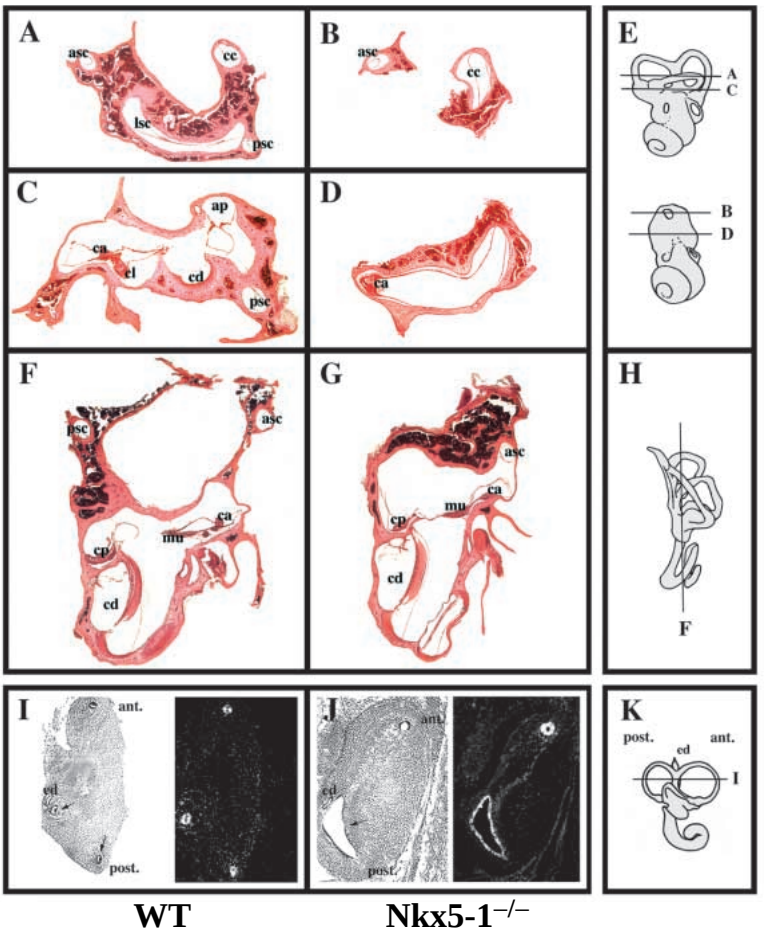

Fig. 3. Individual semicircular canals are differentially affected in the Nkx5-1 knockout mice. Paraffin sections of inner ears of 3-week-old wild-type (A,C,F) and mutant (B,D,G) mice were cut either transversally (A-D) or sagittally (F,G). The levels of sections are shown schematically in E and H. Sections in B and D demonstrate the complete lack of the lateral and posterior semicircular canal. The anterior semicircular canal is less affected. In wild-type mice (A), all three semicircular canals can be easily identified. (C) Cristae of the anterior and lateral semicircular ducts are seen in the wild type, whereas only the crista of the anterior semicircular duct is preserved in the mutant (D). Another pair of ears cut sagittally illustrates that the cochlear morphology is unchanged in the mutant (compare F to G). Again, the anterior semicircular canal is less affected. In this ear, cristae of both semicircular ducts, the posterior and the anterior, are still present. (I,J) A bright-field and corresponding dark-field pictures of sections through ears of E14.5 embryos. The wild-type (I) and mutant (J) ears were sectioned as shown in K and hybridized with a *Nkx5-2* probe. The anterior canal can still be recognized in the mutant, whereas the remaining canals and the crus commune fused into a cystic lumen (arrow in J). Notably, *Nkx5-2* expression persists in the mutant. Abbreviations: ant, anterior; ap, ampulla of the posterior semicircular canal; asc, anterior semicircular canal; ca, crista of the anterior semicircular canal; cc, crus commune; cd, cochlear duct; cl, crista of the lateral semicircular canal; cp, crista of the posterior semicircular canal; ed, endolymphatic duct; lsc, lateral semicircular canal; mu, macula utricle; psc, posterior semicircular canal; post, posterior.

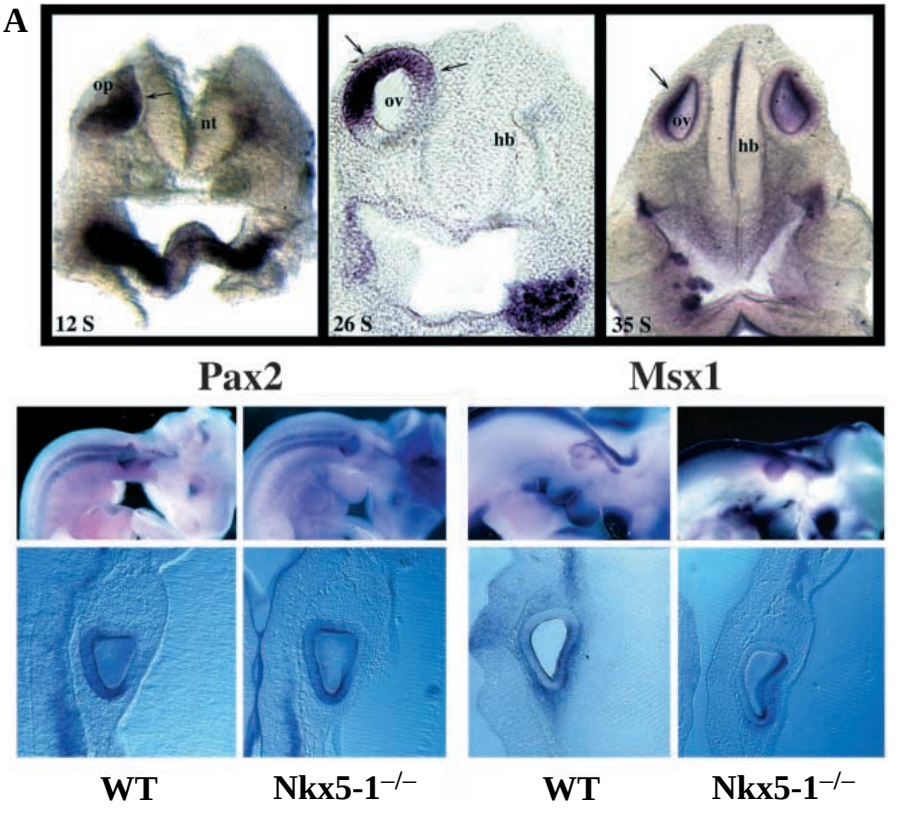

Fig. 4. *Pax2* and *Msx1* expression is unaffected in Nkx5-1$^{-/-}$ mutants. (A) The *Nkx5-1* expression pattern during otic vesicle development is shown on transversal sections. *Nkx5-1* expression domain contacts the hindbrain in placodal (12 somites) and open vesicle stages (not shown) then gradually relocates to a more dorsolateral position (26 somites), and is eventually confined to the lateral wall of the closed vesicle at the 35-somite stage. (B) Whole-mount in situ hybridisations with *Pax2* and *Msx1* in wild-type and Nkx5-1 mutant embryos at 35-somite stage. The approximate planes of corresponding sections are indicated on the scheme. *Pax2* transcripts in wild-type and mutant embryos are confined to the ventromedial wall of the otocyst in a complementary pattern to *Nkx5-1* (A, 35s). The *Msx-1* expression domain overlaps with *Nkx5-1*. Abbreviations: hb, hindbrain; nt, neural tube, op, otic placode; ov, otic vesicle; s, somites.

product could compensate for such hypothetical Nkx5-1 function. Here, the generation and inspection of mice with knockout mutations for both *Nkx5* genes would resolve this question. Compensatory effects or differences in genetic background could also account for the observed variability of the penetrance and expressivity of the phenotype. Interestingly, phenotypic variations have been reported for several hearing deficient mouse mutants (Steel and Harvey, 1992). Similarly, the inactivation of the *int-2* gene resulted in highly variable defects of the inner ear (Mansour et al., 1993).

The early *Nkx5-1* expression in the lateral wall of the otocyst and later in non-sensory epithelium of the vestibular apparatus (Rinkwitz-Brandt et al., 1995, 1996) is in agreement with fate map studies ascribing vestibular cells to the lateral part of the otocyst (Li et al., 1978). The observed Nkx5-1 mutant phenotype of vestibular structures underscores this lineage relationship. However, the differential severity of the mutation on individual semicircular canals cannot be explained simply by the expression pattern of Nkx5-1. It is interesting to note that the order in which the semicircular canals are formed during development (anterior first, then posterior, and the lateral last; Sher, 1971; Martin and Swanson, 1993) correlates inversely with the extent of anatomical malformations. Whether this observation indicates a crucial patterning function of Nkx5-1 predominantly in later developmental events of the semicircular canal formation or reflects its requirement for cell proliferation remains to be investigated. Recent experiments by Fekete et al. (1997) suggest that different mechanisms may play a role in the formation of individual semicircular canals. According to their results, the posterior canal is most severely affected by the blockade of cell death using retrovirally directed overexpression of the human bcl-2 in chick embryos. The effects on the anterior and lateral canal were much less severe. Since the severity of the semicircular canal phenotype of the Nkx5-1 mutants is rather an opposite to what was observed by Fekete et al. (1997), it appears unlikely that Nkx5-1 is involved in the regulation of the cell death during canal morphogenesis.

The agenesis of the cochlea in Pax2 knockout mice (Torres et al., 1996) together with the isolated vestibular phenotype of Nkx5-1 mutants suggest that both organs of the inner ear develop by independent mechanisms and do not interchange critical information during their developmental course. Our finding that *Pax2* expression in Nkx5-1$^{-/-}$ mice is unaltered and confined to the ventromedial wall of the otic vesicle is in keeping with this notion. Thus, both the Nkx5-1 and Pax2 mutant phenotypes reflect the very early lineage separation of epithelial cells in different regions of the otic vesicle. Surprisingly, expression pattern of another gene, *Msx1*, was also unchanged in the Nkx5-1 null mutants, although its expression domain overlaps with that of *Nkx5-1*. This indicates that different control mechanisms may regulate both genes in the same area of the inner ear. However, it is still possible that both genes are expressed in different sets of otocyst cells and this difference simply could not be resolved by means of in situ hybridisation technique. During inner ear organ formation, expression patterns segregate, *Msx1* becoming a marker of the sensory epithelium of all three cristae ampullaris whereas *Nkx5-1* is confined to the non-sensory vestibular epithelium (Wu and Oh, 1996; Rinkwitz-Brandt et al., 1996). Interestingly, a homologous pair of genes, *NK2* and *msh*, in *Drosophila* is expressed in adjacent but non-overlapping domains and both genes display similar but not identical functions during neuronal determination (D'Alessio and Frasch, 1996). Although NK2 and msh are expressed in separate but neighbouring compartments, both are independently involved in regulation of the same downstream genes which belong to the bHLH ACS complex (D'Alessio and Frasch, 1996 and references therein). It remains to be established whether Nkx5-1 and Msx-1 regulate similar genes in different compartments of the mouse inner ear. Until now, however, no detailed description of bHLH gene expression or any inner ear function for the *Msx1* gene have been reported in mammals.

Our in vitro studies on the biochemical properties of the Nkx5-1 protein suggest that it functions as a transcriptional repressor (S. Hoffmann, D. Mennerich, H.-H. Arnold, T. Hadrys and E. Bober, unpublished data). However, no target genes have yet been identified. Similarly, little is known about possible upstream molecules that control *Nkx5-1* gene activity. In this context, it is interesting to note that the Nkx5-1$^{-/-}$ inner ear phenotype closely resembles the fidget mutation, another morphogenetic mouse mutant with deformed vestibular inner ear structures (Truslove, 1956). The *fidget* gene has not been identified but it maps to mouse chromosome 2 (Steel, 1995) which indicates that it is not allelic to the *Nkx5-1* gene located on mouse chromosome 7 (Bober et al., 1994a). Moreover, preliminary experiments suggest that *Nkx5-1* is expressed normally in fidget mutants (S. Rinkwitz-Brandt and E. Bober, unpublished results) suggesting that Nkx5-1 either acts upstream of the *fidget* gene or is involved in a parallel but not overlapping regulatory pathway. Further experiments should clarify the genetic hierarchy of *Nkx5-1* and *fidget* genes and will reveal the cascade of events leading to the establishment of morphogenetic pathways that govern vestibular development.

In summary, our results demonstrate that Nkx5-1 plays an essential role in the formation of the semicircular canals, critical components of the vestibular apparatus. Interestingly, the *Nkx5-1* gene is conserved in sequence and expression in chick, fish and man, and most likely plays a similar role in all vertebrates, indicating a strong evolutionary conservation of the molecular pathways utilized in the formation of vestibular organ (Stadler et al., 1995; H. Herbrand, S. Guthrie, T. Hadrys, S. Hoffmann, H.-H. Arnold, S. Rinkwitz-Brandt, and E. Bober, unpublished results). The Nkx5-1 knockout mouse, therefore, provides an excellent model system to study the molecular basis of vestibular morphogenesis and may apply to a broad range of vertebrate systems.

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