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The role of *Six1* in mammalian auditory system development

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SUMMARY

The homeobox Six genes, homologues to *Drosophila sine oculis* (*so*) gene, are expressed in multiple organs during mammalian development. However, their roles during auditory system development have not been studied. We report that *Six1* is required for mouse auditory system development. During inner ear development, *Six1* expression was first detected in the ventral region of the otic pit and later is restricted to the middle and ventral otic vesicle within which, respectively, the vestibular and auditory epithelia form. By contrast, *Six1* expression is excluded from the dorsal otic vesicle within which the semicircular canals form. *Six1* is also expressed in the vestibuloacoustic ganglion. At E15.5, *Six1* is expressed in all sensory epithelia of the inner ear. Using recently generated *Six1* mutant mice, we found that all $Six1^{+/-}$ mice showed some degree of hearing loss because of a failure of sound transmission in the middle ear. By contrast, $Six1^{-/-}$ mice displayed malformations of the auditory system involving the outer, middle and inner ears. The inner ear development in $Six1^{-/-}$ embryos arrested at the otic vesicle stage and all components of the inner ear failed to form due to increased cell death and reduced cell proliferation in the otic epithelium. Because we previously reported that *Six1* expression in the otic vesicle is *Eya1* dependent, we first clarified that *Eya1* expression was unaffected in $Six1^{-/-}$ otic vesicle, further demonstrating that the *Drosophila* Eya-Six regulatory cassette is evolutionarily conserved during mammalian inner ear development. We also analyzed several other otic markers and found that the expression of *Pax2* and *Pax8* was unaffected in $Six1^{-/-}$ otic vesicle. By contrast, *Six1* is required for the activation of *Fgf3* expression and the maintenance of *Fgf10* and *Bmp4* expression in the otic vesicle. Furthermore, loss of *Six1* function alters the expression pattern of *Nkx5.1* and *Gata3*, indicating that *Six1* is required for regional specification of the otic vesicle. Finally, our data suggest that the interaction between *Eya1* and *Six1* is crucial for the morphogenesis of the cochlea and the posterior ampulla during inner ear development. These analyses establish a role for *Six1* in early growth and patterning of the otic vesicle.

Key words: *Six1*, Auditory system, Inner ear, Regional specification, Mouse, *Eya1*, *Pax2*, *Fgf3*, *Fgf10*, *Bmp4*, *Nkx5.1*, *Gata3*

INTRODUCTION

The mammalian auditory system includes three distinct parts: the outer, the middle and the inner ears. Despite the complexity and multiple functions, the ear forms one anatomical unit that serves both hearing and equilibrium. The earliest morphological evidence for inner ear development is the otic placode, a thickened area of surface ectoderm on each side of the hindbrain. The otic placode invaginates to form the otic cup and vesicle, which subsequently undergoes proliferative growth and eventually differentiates into different regions of the inner ear. In the mouse, the basic architecture of the inner ear is fully established by E14.5 (Morsli et al., 1998).

A large number of otic genes, including transcription factors, secreted factors, receptors, cell adhesion proteins and others have been described; however, functional importance in early morphogenetic processes has only been demonstrated for some genes (Fekete and Wu, 2002). The homeobox-containing genes such as the NK-related homeobox gene *Nkx5.1* and the paired-box gene *Pax2* are expressed in complementary patterns in the otic vesicle, with *Nkx5.1* dorsolaterally and *Pax2* ventromedially (Herbrand et al., 1998). Mutation in the *Nkx5.1* gene results in agenesis of the semicircular canals and circling behavior (Hadrys et al., 1998), while mutation in the *Pax2* gene leads to agenesis of the cochlea (Torres et al., 1996). The GATA family zinc-finger gene *Gata3* shows reciprocal relationships with *Pax2* in the regional patterning of the early otocyst and cellular patterning within the sensory epithelia and ears of *Gata3*-null mouse mutants remain cystic, with a single extension of the endolymphatic duct (Karis et al., 2001; Lawoko-Kerali et al., 2002). The eyes absent gene *Eya1*, which encodes a transcription coactivator, is also expressed early in the otic epithelium and the inner ear development in *Eya1* knockout mice arrests at the otic vesicle stage (Xu et al., 1999a). This is the first described mouse mutant lacking all sensory areas of the inner ear. Secreted factors like the Bmp-family of Tgfβ-like polypeptides, Fgfs and receptor molecules like the Fgfr2 IIIb and Fgfr1 are also expressed in the otic epithelium and serve as signaling molecules in early otic development (Chang et al., 1999; Ohuchi et al., 2000; Pirvola et al., 2000; Noramly and Grainger, 2002; Pirvola et al., 2002). Nonetheless, it is largely unknown how these genes function

and respond to the inductive signals from neighboring tissues in the morphogenetic processes of inner ear development.

The murine homeobox Six gene family has been identified on the basis of sequence homology with the *Drosophila sine oculis* (*so*) gene. At present, six members (*Six1-Six6*) of the Six gene family have been isolated and they are suggested to interact with Pax and Eya genes based on their wide co-expression in many tissues during mammalian organogenesis and development (Oliver et al., 1995a; Oliver et al., 1995b; Kawakami et al., 1996; Chen et al., 1997; Pignoni et al., 1997; Xu et al., 1997a; Xu et al., 1997b). However, their functional roles during mammalian inner ear development have not been studied. In this study, we analyzed the expression of *Six1* during inner ear development and its role in mouse auditory system development. In the developing inner ear, *Six1* is expressed in all sensory epithelia. Inactivation of the *Six1* gene led to malformation of the auditory system involving the outer, middle and inner ears. The inner ear development in $Six1^{-/-}$ embryos arrested at the otic vesicle stage and all components of the inner ear failed to form because of increased cell death and reduced cell proliferation in the otic epithelium. Molecularly, *Six1* is not required for the expression of *Eya1*, *Pax2* and *Pax8* in the otic epithelium. By contrast, *Six1* is required for the normal expression of *Fgf3*, *Fgf10*, *Bmp4*, *Gata3* and *Nkx5.1* in the otic vesicle, indicating that *Six1* is required for the regional specification of the otic vesicle. Finally, we provide evidence for a genetic interaction between *Eya1* and *Six1* during inner ear development. These analyses indicate that similar to *Eya1*, *Six1* is not required for the initiation of otic placode morphogenesis to form otic vesicle, but is required for the normal growth and regional specification of the otic vesicle.

MATERIALS AND METHODS

Animals and genotyping

Eya1/Six1 double heterozygous mice were generated by crossing mice carrying mutant alleles of *Eya1* and *Six1* ($Six1^{lacZ}$) and genotyping of mice and embryos was performed as previously described (Torres et al., 1995; Xu et al., 1999a; Xu et al., 2002; Laclef et al., 2003).

ABR testing and ear morphologic analyses

We used a computer-assisted evoked potential system to obtain ABR thresholds for tone pips at 5, 8, 11, 16, 22, 32 and 45 kHz (tone pip duration 5 mseconds); repetition rate 30/secons and averaged responses to 512 pips of alternating polarity.

Adult ears were sectioned after paraffin wax embedding (8 μm) for morphological analysis as described (Xu et al., 1999a). We examined 10 heterozygotes in both 129/Sv and C57BL/6J backgrounds and compared them with sections from five 129/Sv and three C57BL/6J wild-type mice.

The latex paintfilling of the ears at E16.5 and 17.5 was performed as described (Morsli et al., 1998). The paintfilled inner ears were dissected out and photographed.

Phenotype analyses and in situ hybridization

Embryos for histology and in situ hybridization were dissected out in PBS and fixed with 4% paraformaldehyde (PFA) at 4°C overnight. Embryonic membranes were saved in DNA isolation buffer for genotyping. Histology was performed as described (Xu et al., 1999a). To visualize $Six1^{lacZ}$ expression, mutant embryos were stained with X-gal and sectioned as described (Xu et al., 2002). To reveal the middle ear ossicles, we performed skeletal staining of cartilage and bone as described (Peters et al., 1998).

For in situ hybridization, we used four wild-type or mutant embryos at each stage for each probe as described (Xu et al., 1997a).

TUNEL assay and BrdU labeling

TUNEL assay was performed as described (Xu et al., 1999a). To label the proliferating cells, timed pregnant mice at E8.5 and 9.5 were injected i.p. twice at 2-hour intervals with 5-bromodeoxyuridine (BrdU, Sigma) and embryos were collected as described (Xu et al., 1999b). Paraffin wax embedded sections of 6 μm were prepared and denatured with 4N HCl for 1 hour at 37°C. Mouse anti-BrdU monoclonal antibody and goat anti-mouse IgG coupled with HRP or Cy3 were used for detection. The number of proliferating cells was counted in serial sections from each otic placode or vesicle, and at least five embryos (10 ears) of each genotype were counted.

RESULTS

Six1 expression in the developing inner ear

As the inserted *lacZ* transgene displayed an expression pattern identical to the *Six1* mRNA distribution obtained by in situ hybridization, we analyzed the expression of *Six1* gene during inner ear development in $Six1^{lacZ}$ heterozygotes by staining for β-galactosidase activity (Fig. 1). During inner ear development, *Six1* expression was first detected in the ventral region of the otic pit at around E8.75 (Fig. 1A). Its expression domain expands as otic development proceeds and by E9.5, *Six1* expression became restricted to the middle and ventral otic vesicle within which the vestibular and auditory epithelia form respectively (Fig. 1B,C). By contrast, *Six1* expression is excluded from the dorsal region within which the semicircular canals form (arrows, Fig. 1A-C). *Six1* is also expressed in the vestibuloacoustic ganglion (gVIII, Fig. 1D). During subsequent stages of inner ear development, *Six1* is expressed in all sensory epithelia. In the vestibule, *Six1* is expressed in the developing cristae, saccule and utricle (Fig. 1D-F). In the utricle and saccule, *Six1* is expressed throughout the neuroepithelium at E12.5 (Fig. 1D and data not shown) and by E15.5 its expression appears to be restricted to the hair cells in the middle of the epithelium (Fig. 1F). In the cochlea, *Six1* is expressed throughout the future greater and lesser epithelial ridge (GER and LER) of the cochlear duct at E12.5 (Fig. 1G); however, its expression became weaker in a region within which the organ of Corti begins to differentiate at this stage (bracket, Fig. 1G). In mice, the progenitors of hair and supporting cells in the primordial organ of Corti become postmitotic between E12.5 and E14.5 (Ruben, 1967). At E14.5-E15.5 when the cochlear duct has made 1.5 turns, hair and supporting cell differentiation initiates in the mid-basal region of the cochlea and hair cell differentiation proceeds until the entire length of the sensory epithelium is patterned into one inner row and three outer rows of hair cells at E17.5-E18.5 (Sher, 1971; Lim and Anniko, 1985; Chen et al., 2002). In the apex of E15.5 cochlea, *Six1* expression was weakly detected in the supporting cells and started to appear in the inner hair cell (arrowhead, Fig. 1H). By contrast, strong *Six1* expression was observed in the GER and LER flanking the developing organ of Corti. In the basal cochlea where the development of the organ of Corti is more advanced than in the apex, *Six1* is expressed in the outer and inner hair cells (arrows and

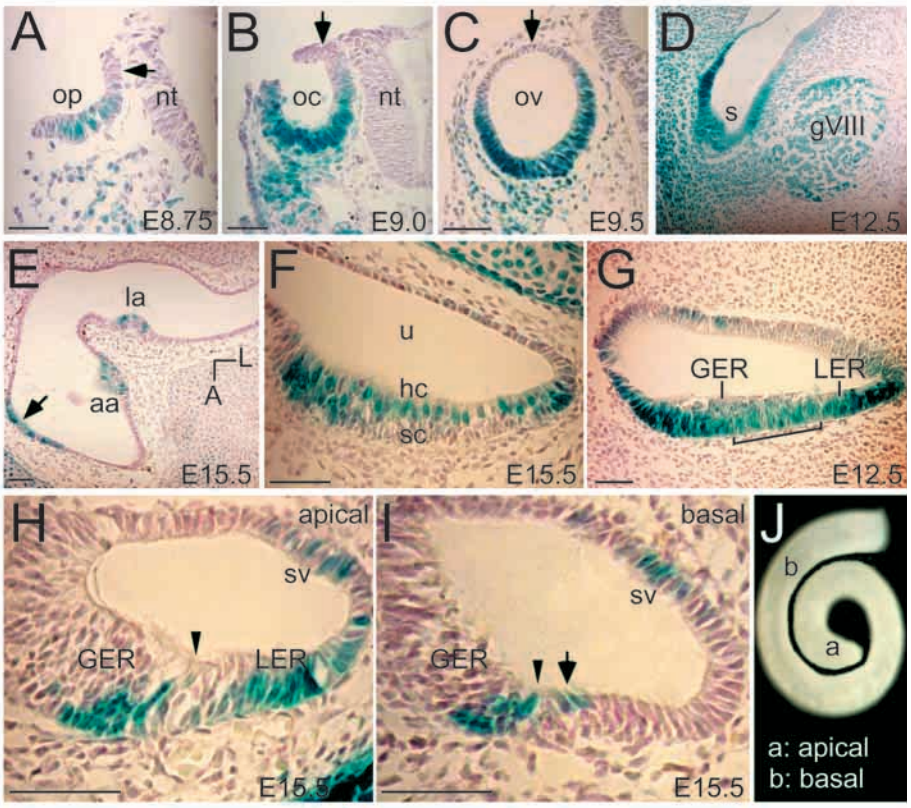

Fig. 1. *Six1* expression during inner ear development. E8.75 to 15.5 $Six1^{lacZ}$ heterozygous embryos or inner ears were stained with X-gal for $Six1^{lacZ}$ and sectioned through the inner ear region. (A) A transverse section showing *Six1* expression in the otic pit (op) at E8.75. (B) A transverse section showing Six1 expression in the otic cup (oc) at E9.0. (C) A transverse section showing *Six1* expression in the middle and ventral otic vesicle (ov) at E9.5. Note *Six1* is excluded from the dorsal otic pit and vesicle (arrows). (D) A transverse section at E12.5 showing *Six1* expression in the saccular region and the vestibuloacoustic ganglion (gVIII). For A-D, dorsal is upwards. (E) A section showing *Six1* expression in the primordia of lateral (la) and anterior (aa) Crista ampullaris. Arrow indicates the origin of the anterior ampulla on the other side. (F) A section showing *Six1* expression in the hair cells (hc) of the utricle (u). (G) In the cochlea, *Six1* is expressed throughout the future greater and lesser epithelial ridge (GER and LER). Note its expression level is reduced in an area that will become the organ of Corti (bracket) at E12.5. (H) In E15.5 cochlea, *Six1* is expressed weakly in the supporting cells and first appeared in the inner hair cell (arrowhead) in the apex. Strong *Six1* expression was also observed in the cells flanking the developing organ of Corti in the GER and LER. (I) In the base of E15.5 cochlea, *Six1* is expressed in the outer (arrow) and inner (arrowhead) hair cells. It is also expressed in some cells in the GER. In addition, it is expressed in the thinner part of the cochlea duct that will probably differentiate into the stria vascularis (sv). (J) A latex paintfilled E15.5 cochlea showing the apical and basal regions. Scale bars: 50 μm.

arrowhead, Fig. 1I), but was undetectable in the supporting cells beneath the hair cells. Strong *Six1* expression was maintained in some cells in the GER. In addition, *Six1* is expressed in the region from which the stria normally develops. Therefore in the developing organ of Corti, *Six1* is initially expressed in the progenitors of hair and supporting cells but its expression disappears when the progenitor cells exit cell cycle and later on it is expressed in the terminally differentiated hair cells. Taken together, our data show that *Six1* is predominantly expressed in all sensory regions of the inner ear, suggesting a role for *Six1* in the morphogenesis of sensory organs during mammalian inner ear development.

$Six1^{+/-}$ mice show a conductive hearing loss

Most of the 129 *Six1* heterozygous mice had certain degree of hearing loss, as determined by auditory-evoked brainstem response (ABR) threshold measurements (n=26; Fig. 2A). Of the 13 $Six1^{+/-}$ mice analyzed, eight had hearing loss in both ears, two had hearing loss in one ear and three showed mild hearing loss in both ears. Similar observation was obtained in C57BL/6J background.

To determine whether the hearing loss is conductive, sensorineural or both, we sectioned the $Six1^{+/-}$ ears associated with hearing loss. All $Six1^{+/-}$ ears with hearing losses revealed middle ear abnormalities: typically, a failure of the ossicles to complete a sound transmission path from the tympanum to the oval window. Although the stapes attached to oval window, the VIIth cranial nerve passed abnormally between the oval window and stapedial artery or over the surface of the cochlea under the stapedial artery (Fig. 2C,D and data not shown). The middle ears also showed morphologically abnormal ossicles, including stapes with a small lumen and the middle ear space was filled up with loose connective tissues (arrow, Fig. 2F and data not shown). The latter could be due to secondary inflammation. The middle ear space appeared to be small (Fig. 2C,D,F,G) and the tympanic membrane was also abnormal (arrowheads, Fig. 2F,G). In four cases from 3 animals, there were no stapedial arteries (data not shown). In the inner ear, the cochlear spiral of all heterozygotes was well formed, although four out of 22 $Six1^{+/-}$ ears (three out of 11 embryos) showed slightly shortened cochlea by latex paintfilling at E16.5 (see Fig. 8 and Table 1). Nonetheless, our analyses clearly show that there is a failure of sound transmission in the middle ear.

Six1 is required for normal growth of the otic vesicle

Auditory system abnormalities in *Six1* homozygotes involve the outer, middle and inner ears (Fig. 3). The outer ear revealed malformed auricles, preauricular pits and malformed eardrums (Fig. 3A,B and data not shown). In the middle ear, the incus was present but malformed or fused with the malleus (arrowheads, Fig. 3D). The short process of the malleus was typically absent (arrow, Fig. 3D), as was the stapes, while the tympanic cavity is present in *Six1* homozygotes (data not shown). In the inner ear, although the otic vesicle forms, it appears to be smaller and abnormal at E10.5 (arrow, Fig. 3F). By E12.5, no inner ear

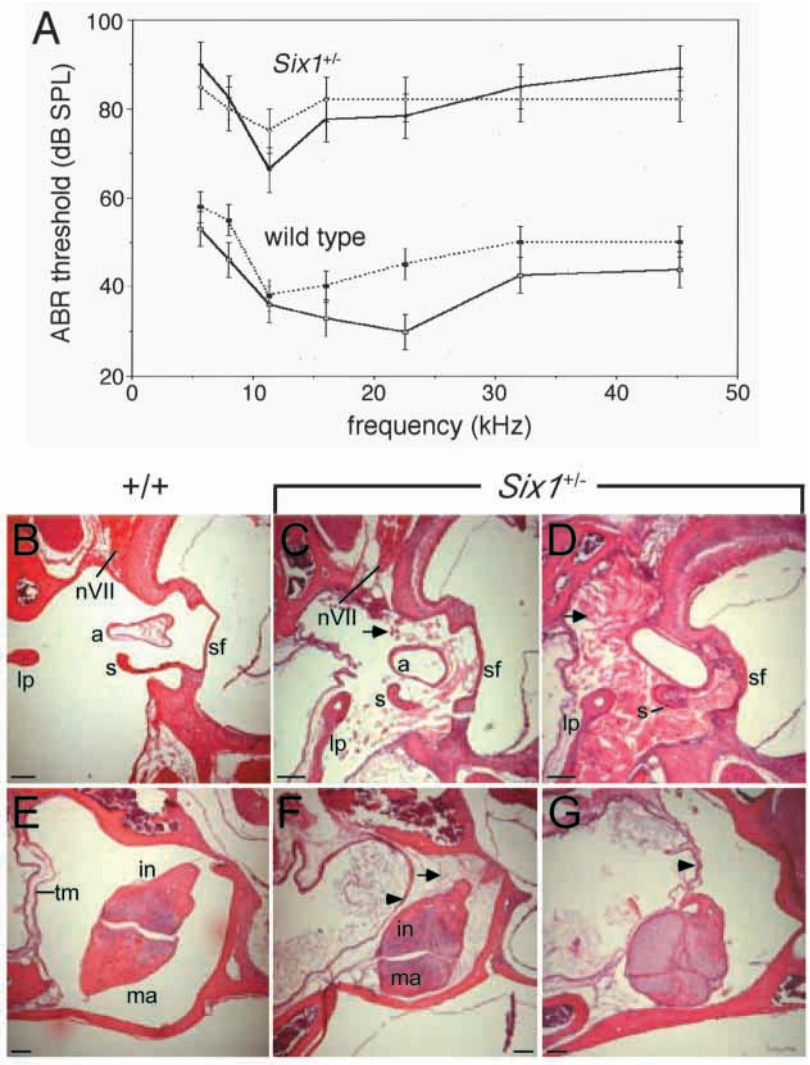

Fig. 2. ABR threshold measurements and pathological structures in *Six1*$^{+/-}$ adult mouse ears. (A) Average threshold±s.e.m. for wild-type and *Six1*$^{+/-}$ ears in backgrounds 129/Sv and C57BL/6J (129/Sv, *n*=6 wild type, 13 *Six1*$^{+/-}$; C57BL/6J, *n*=6 wild type, 10 *Six1*$^{+/-}$). Averages used 90 dB SPL for thresholds beyond the upper limit of the sound system. The hearing loss was variable among mice and ears. Of the 13 *Six1*$^{+/-}$ mice tested, eight mice had severe hearing loss in both ears (threshold shifted by 50 dB between 15 and 32 kHz), two mice had mild hearing loss in both ears (threshold shifted by 20 dB), whereas three mice had normal hearing in the right ears and >70 dB loss in the left ears. Broken lines, C57BL/6J strain; unbroken lines, 129/Sv strain. (B-G) Histological analysis in wild type and *Six1*$^{+/-}$ ears. (B) Wild-type ear showing the stapes seated in the oval window (not visible because apposed by the stapes footplate, sf), and one arch of the stapes (s). The stapedial artery (a) is normally positioned. Part of the long process of the malleus (lp) is also present in this section. nVII, the VIIth cranial nerve. (C,D) *Six1*$^{+/-}$ ears showing that the footplates of the stapes are seated in the oval window. However, the VIIth nerve passed abnormally close to the oval window and the stapes or filled up the middle ear space near the oval window (arrows). Part of the stapes arches and the long process of the malleus are present in these sections. (E) Wild-type superior region of the middle ear space showing the junction of the malleus (ma) and incus (in). tm, tympanic membrane. (F,G) *Six1*$^{+/-}$ superior region of the middle ears showing the junction of the malleus and incus. The middle ear space was small and partially filled with loose connective tissue (arrow). The tympanic membrane was also abnormal (arrowheads). ABR threshold from these ears demonstrated a severe hearing loss. Scale bars: 100 μm.

Table 1. Inner ear defects in *Eya1* and *Six1* heterozygous embryos

	Six1$^{+/-}$ *n*=22 (11)		*Eya1*$^{+/-}$ *n*=22 (11)		*Six1*$^{+/-}$/*Eya1*$^{+/-}$ *n*=40 (20)	
Abnormalities	*n*	%	*n*	%	*n*	%
Endolymphatic duct (truncated)	2 (2)	9.1 (18.2)	3 (2)	13.6 (18.2)	5 (4)	12.5 (20)
Endolymphatic sac (absent or mal-shaped)	5 (3)	22.7 (27.3)	8 (5)	36.4 (45.5)	15 (9)	37.5 (45)
Saccule (malformed)	4 (3)	18.2 (27.3)	0	0	7 (5)	17.5 (25)
Posterior ampulla (absent)	0	0	0	0	7 (5)	17.5 (25)
Posterior semicircular canal (absent or truncated)	0	0	0	0	7 (5)	17.5 (25)
Cochlea* (shortened)	4 (3)	18.2 (27.3)	4 (4)	18.2 (36.4)	19 (13)	47.5 (65)

n, number of ears (the numbers shown in parentheses are the numbers of embryos).
%, the percentage of the ears or the embryos (shown in the parentheses) that showed defects.
*Four out of 22 *Six1*$^{+/-}$ 129 ears (3 of 11 embryos) showed slightly shortened cochlea at E16.5, three completed near 1.5 turns and one completed between 1 and 1.25 turns instead of 1.75 turns (Fig. 8B). Similarly, four out of 22 *Eya1*$^{+/-}$ 129 ears (4 of 11 embryos) showed slightly shortened cochlea, two reached near 1.5 turns and the other two coiled between 1 and 1.25 turns at E16.5. By contrast, 19 of 40 *Eya1*/*Six1* 129 compound heterozygous ears (13 out of 20 embryos) showed severely affected cochlea with abnormal distal tips. Among the 19 affected cochlea, 16 completed less than one turn and three coiled between 1 and 1.25 turns.

structure or sometimes only severely malformed vestibule-like structure was observed in *Six1*$^{-/-}$ embryos (arrow, Fig. 3H). The malformed vestibule-like structure observed in some *Six1*$^{-/-}$ embryos at E12.5 also failed to develop further (data not shown). In addition, the vestibuloacoustic (gVIII) and petrosal (gIX) ganglia were absent (asterisk and arrowhead, Fig. 3H). Thus, *Six1* plays a direct role in the normal development of the mammalian auditory system.

The failure of inner ear development in *Six1*$^{-/-}$ embryos was associated with an increased cell death as detected by TUNEL analysis. Although apparent morphological difference of the otic vesicle was not observed between *Six1*$^{-/-}$ and wild-type

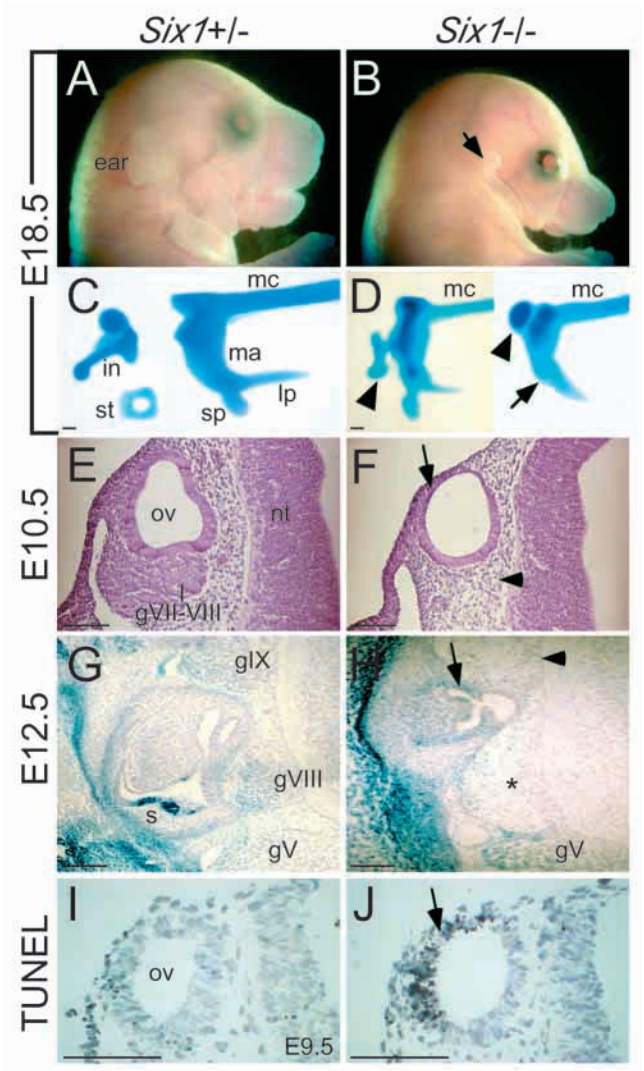

Fig. 3. Auditory system development in *Six1* homozygotes. (A,B) All *Six1* homozygotes die at birth and exhibit severe auditory system defects involving the outer (arrow), middle and inner ears, as well as other defects. (C,D) Microdissected middle ear ossicles from E18.5 wild-type and *Six1*$^{-/-}$ embryos. In the mutant, the incus (in) is malformed and fused with the malleus (ma) (arrowheads) and the stapes (st) is absent. The short process (sp) of the malleus is often missing (arrow) and the long process (lp) is also shortened. (E,F) Transverse sections of E10.5 *Six1* heterozygous and homozygous embryos stained with Hematoxylin and Eosin showing the developing otic vesicle (ov) and the vestibuloacoustic ganglion (gVIII). In *Six1*$^{-/-}$ embryos, although the otic vesicle formed, it appeared much smaller and abnormal (arrow) and the gVIII is absent (arrowhead). (G,H) Transverse sections of E12.5 wild-type and *Six1* mutant embryos stained with X-gal for *Six1*^{lacZ} and counterstained with diluted Hematoxylin showing the developing inner ear, *Six1*^{lacZ} expression in the utricle and saccule region, semicircular canals, cranial ganglia gIX, gVIII and gV in the heterozygotes. However, in *Six1*$^{-/-}$ embryos, only malformed semicircular canal-like structure was observed (arrow). Other inner ear structures are not formed and gIX (arrowhead) and gVIII (asterisk) are absent in the homozygotes. (I,J) TUNEL analysis of transverse sections through the ear region of *Six1*$^{+/-}$ and *Six1*$^{-/-}$ embryos at E9.5. Numerous apoptotic cells are only detected in the lateral wall of *Six1*$^{-/-}$ otic vesicle (arrow). Scale bars: 100 μm.

embryos at E9.5 (data not shown), numerous apoptotic cells in the lateral wall of *Six1*$^{-/-}$ otic vesicle were detected (arrow, Fig. 3J). At E10.5, apoptotic cells were also increased in the medial region of *Six1*$^{-/-}$ otic vesicle (data not shown). Thus, *Six1* is required for otic epithelial cell survival.

We next tested whether *Six1*$^{-/-}$ otic epithelial cells proliferate appropriately by assaying BrdU incorporation in the mutant otic placode and vesicle at E8.5 and 9.5, before apparent morphological alteration was seen in *Six1*$^{-/-}$ embryos. Four hours after BrdU injection, BrdU-labeled cells were seen throughout the otic placode in wild-type embryos (Fig. 4A). However, in *Six1*$^{-/-}$ embryos, the number of BrdU-labeled cells was reduced in the otic placode (arrowhead, Fig. 4B). By E9.5, BrdU-positive cells were largely reduced in the dorsal half of *Six1*$^{-/-}$ otic vesicle (above arrowheads, Fig. 4D). Using an image analysis system, we next counted the number of BrdU-positive cells from 10 wild-type and 10 *Six1*$^{-/-}$ ears at each stage on serial sections to determine the labeling index (Fig. 4E). At E8.5, the number of BrdU-positive cells in *Six1*$^{-/-}$ otic placode was 80% of wild-type embryos (Fig. 4E). By E9.5, the number of BrdU-positive cells in *Six1*$^{-/-}$ otic vesicle was reduced to 50% of that in wild-type embryos (Fig. 4E). As the epithelial cells in the lateral wall of *Six1*$^{-/-}$ otic vesicle undergo abnormal apoptosis from E9.5 (Fig. 3J), to further clarify whether the reduction of cell proliferation in E9.5 *Six1*$^{-/-}$ otic vesicle is due to abnormal cell death, we determined the labeling index from the lateral and medial half of the otic vesicle, respectively. In the lateral half, the number of BrdU-labeled cells in *Six1*$^{-/-}$ otic vesicles was 60% of that in wild-type embryos (E9.5L, Fig. 4E). Similarly, in the medial half of the otic vesicle, although no abnormal apoptosis was observed in *Six1*$^{-/-}$ embryos at E9.5, the number of BrdU-labeled cells was reduced to 40% of that seen in wild-type embryos (E9.5M, Fig. 4E). Thus, *Six1* is required for normal growth of the otic vesicle by regulating cell proliferation during early otic development.

Eya1, *Pax2* and *Pax8* expression does not require *Six1* function during early otic development

To determine the molecular defects in early otic development of *Six1*$^{-/-}$ animals, we first examined whether the expression of the Eya and Pax gene families depends upon *Six1*. Studies in *Drosophila* indicate that *eya* is epistatic to *so* and both genes reside within the same genetic and molecular pathway downstream of the *Pax6* gene *ey* (Halder et al., 1998). As we previously found that *Six1* mRNA expression was undetectable in *Eya1*$^{-/-}$ embryos (Xu et al., 1999a), we first analyzed the *Six1*^{lacZ} expression by X-gal staining to further confirm this observation. *Six1*^{lacZ} expression was also undetectable in *Eya1*$^{-/-}$ otic epithelium, further demonstrating that *Six1* expression in the otic epithelium is *Eya1* dependent (Fig. 5A,B). We next analyzed the expression of *Eya1* in *Six1*$^{-/-}$ embryos to further clarify their regulatory relationship during early otic development. *Eya1* is normally co-expressed with *Six1* in the otic epithelium and its expression was unaffected in *Six1*$^{-/-}$ otic vesicles at E9.5 and E10.5 (Fig. 5C,D and data not shown). This further confirms that *Eya1* functions upstream of *Six1* and that the Eya-Six regulatory pathway elucidated in *Drosophila* eye imaginal disc is evolutionarily conserved in early mammalian otic development. Because we previously found that the expression of both *Pax2* and *Pax8* was

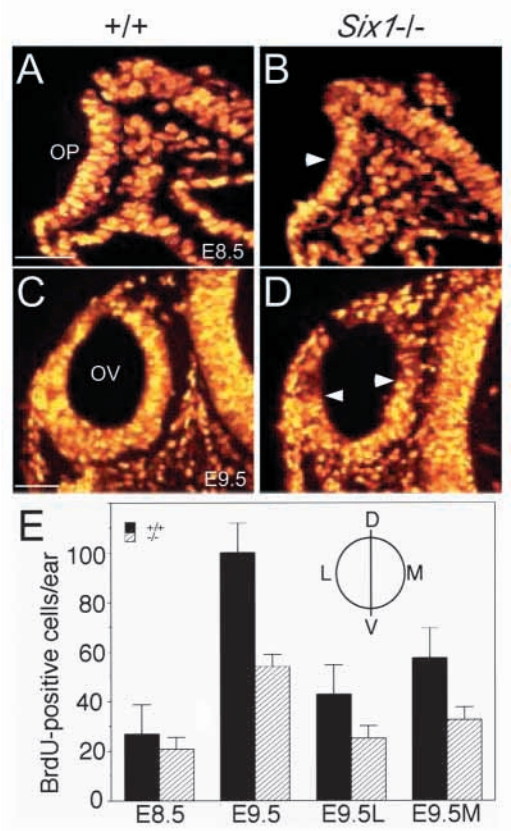

Fig. 4. *Six1* controls proliferation of otic epithelial cells during early inner ear development. (A-D) Transverse sections of otic regions from E8.5 (A,B) and E9.5 (C,D) wild-type and *Six1*$^{-/-}$ embryos showing BrdU-labeled cells (orange). BrdU-positive cells were slightly reduced in *Six1*$^{-/-}$ otic placode (OP) at E8.5 (B) and by E9.5, BrdU-positive cells were largely reduced in the dorsal region of *Six1*$^{-/-}$ otic vesicle (OV, above arrowheads in D). (E) The labeling index was determined by counting the number of BrdU-positive cells from each otic placode or vesicle. Ten ears for each genotype were counted and the numbers were averaged. At E8.5, the number of BrdU-labeled cells in *Six1*$^{-/-}$ otic placode was 80% of wild-type embryos. By E9.5, the number of BrdU-labeled cells in *Six1*$^{-/-}$ otic vesicle was reduced to 50% of that in wild-type embryos. In *Six1*$^{-/-}$ embryos, the number of BrdU-positive cells in the lateral otic vesicle was reduced to 60% (E9.5L), while in the medial half the number was reduced to 40% of that in wild-type embryos (E9.5M). Scale bars: 50 μm.

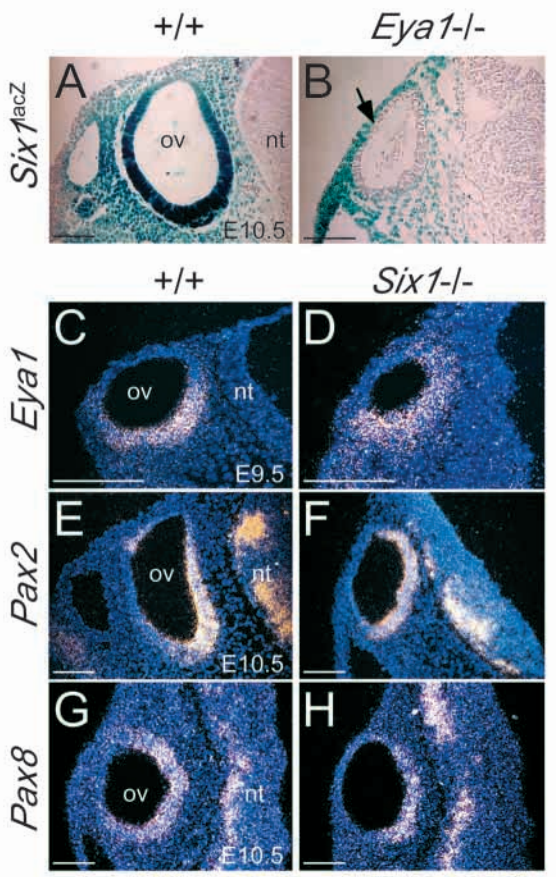

Fig. 5. *Eya1*, *Pax2* and *Pax8* expression in otic epithelium is *Six1*-independent. (A,B) *Six1*^{lacZ} is normally expressed in otic vesicle (ov) and its expression was undetectable in *Eya1*$^{-/-}$ embryos. (C-H) *Eya1* (C,D), *Pax2* (E,F) and *Pax8* (G,H) expression levels in otic vesicle were unaffected in *Six1*$^{-/-}$ embryos. Scale bars: 100 μm. nt, neural tube.

unaffected in *Eya1*$^{-/-}$ otic epithelium (Xu et al., 1999a), we next examined whether the Pax gene expression in the otic epithelium is also *Six1* independent. In *Six1*$^{-/-}$ embryos, *Pax2* and *Pax8* are expressed in the otic placode and vesicle at normal levels at E8.5-E10.5 (Fig. 5E-H and data not shown). This indicates that similar to *Eya1*, *Six1* is also not required for the expression of both *Pax2* and *Pax8* in the otic epithelium.

Six1 is required for the activation of *Fgf3* expression and the maintenance of *Fgf10* and *Bmp4* expression in the otic vesicle

We next examined the expression of several other well-characterized molecular markers in the otic epithelium at E8.5-10.5. *Fgf3*, a member of the *Fgf* superfamily of secreted signals, begins to be expressed in the ventrolateral wall of the otic vesicle at E9.5 and in the delaminating neuroblasts and gVIII (Fig. 6A and data not shown). Inactivation of *Fgf3* results in inner ear defects and its expression in the otic vesicle is *Eya1* dependent (Mansour et al., 1993; Xu et al., 1999a). Similarly, *Fgf3* expression was undetectable in *Six1*$^{-/-}$ otic vesicle from E9.5 (Fig. 6B). By contrast, its expression in the hindbrain was relatively normal in *Six1*$^{-/-}$ embryos (Fig. 6A,B). *Fgf10*, another member of the Fgf superfamily, is expressed in the otic placode and vesicle and by E10.5, its expression became concentrated to a broad region in the ventral half, a small patch in the posterodorsal wall and in the neuroblast cells and gVIII (Fig. 6C) (Pirvola et al., 2000). The expression of both *Fgf3* and *Fgf10* in the ventrolateral wall of the otic vesicle and in the neuroblasts and gVIII overlaps and both genes are suggested to function through their receptor *Fgfr2 IIIb* (Pauley et al., 2003). When both are knocked out, the otic vesicle fails to form (Wright and Mansour, 2003). In *Six1*$^{-/-}$ embryos, *Fgf10* expression was unaffected in the otic placode (data not shown). However by E10.5, residual *Fgf10* expression was only observed in the ventromedial wall of *Six1*$^{-/-}$ otic vesicle (arrow, Fig. 6D). The expression of both *Fgf3* and *Fgf10* was undetectable in the gVIII in *Six1*$^{-/-}$ embryos, further confirming the absence of this structure in *Six1* homozygotes. *Bmp4*, a member of the Tgfβ superfamily, is expressed in a

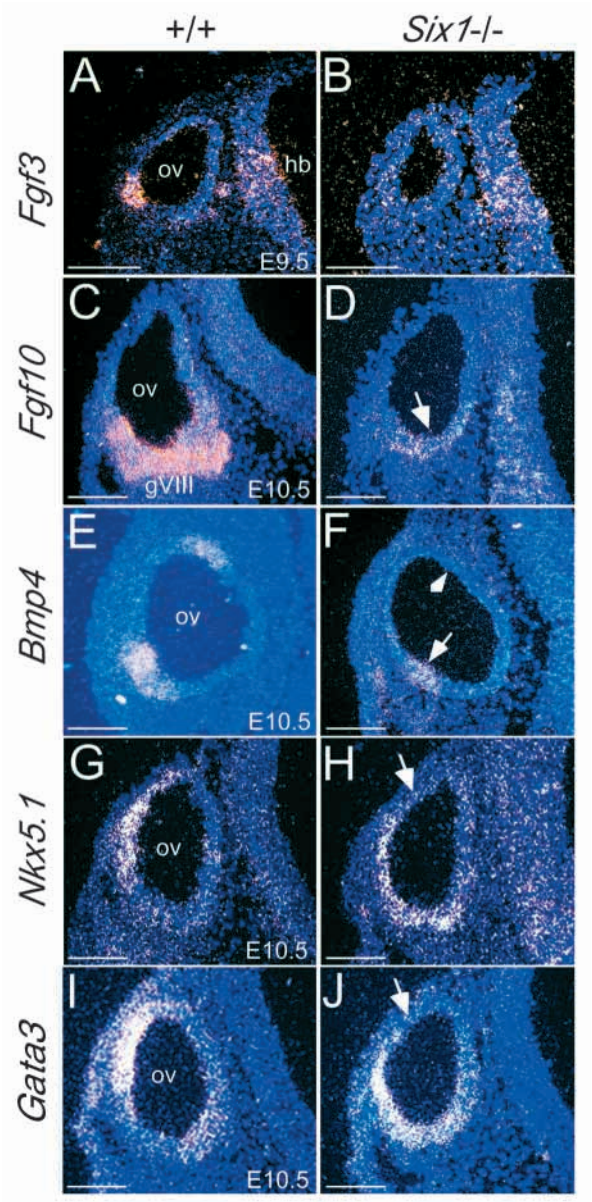

Fig. 6. Loss of *Six1* function alters the expression of *Fgf3*, *Fgf10*, *Bmp4*, *Nkx5.1* and *Gata3* in the otic vesicle. (A-D,G-J) Transverse sections; (E,F) Horizontal sections. (A,B) *Fgf3* begins to be expressed in the ventrolateral region of the otic vesicle (ov) at E9.5 and its expression was undetectable in *Six1*$^{-/-}$ embryos. Note *Fgf3* expression in the hindbrain (hb) is relatively normal in *Six1*$^{-/-}$ embryos. (C,D) *Fgf10* is normally expressed in a broad region of ventral otic vesicle and the gVIII in wild-type embryos at E10.5; however, residual expression of *Fgf10* was only detected in the ventromedial otic vesicle in *Six1*$^{-/-}$ embryos (arrow). (E,F) *Bmp4* is normally observed in two specific spots, one dorsal and one ventrolateral in the otic vesicle of wild-type embryos at E10.5. In *Six1*$^{-/-}$ embryos, the dorsal expression spot (arrowhead) was undetectable and the ventrolateral spot became very weak (arrow). (G,H) *Nkx5.1* is expressed in the dorsolateral region of the otic vesicle in wild-type embryos at E10.5. In *Six1*$^{-/-}$ embryos, however, *Nkx5.1* expression is excluded from the dorsolateral region (arrow) and its expression domain shifted or expanded ventrally, including the ventral-most region. (I,J) *Gata3* is expressed strongly dorsolaterally and weakly ventromedially in the wild-type embryos at E10.5. Similarly, in *Six1*$^{-/-}$ embryos it is not expressed in the dorsolateral region (arrow) and its expression domain shifted ventrally. By contrast, its expression in the ventromedial region of the otic vesicle was unaffected in *Six1*$^{-/-}$ embryos. Scale bars: 100 μm.

broad region of the lateral otic vesicle at E9.5 and by E10.5, its expression is restricted to two patches, one in the dorsal and the other in the lateral region of the otic vesicle which mark the sensory anlagen of the cristae (Fig. 6E) (Wu and Oh, 1996). In *Six1*$^{-/-}$ embryos, *Bmp4* was expressed in the otic vesicle at E9.5 but its expression significantly diminished at E10.5 with the dorsal expression domain disappeared and the lateral domain weakened greatly (arrowhead and arrow, Fig. 6F). Alteration of *Fgf3*, *Fgf10* and *Bmp4* expression in *Six1*$^{-/-}$ embryos could indicate that *Six1* regulates the transcription of these genes or that *Six1* is required for the specification of the cells that express these genes or both. Nonetheless, these results indicate that *Six1* is required for the normal expression of *Fgf3*, *Fgf10* and *Bmp4* in the otic vesicle.

Loss of *Six1* function alters the expression profile of *Nkx5.1* and *Gata3* in the otic vesicle

During mouse inner ear development, the semicircular canals form from the dorsal region of the otic vesicle, the vestibular epithelia form from the middle region and the auditory epithelia form from the ventral region of the otic vesicle, respectively (Li et al., 1978). Because only severely affected semicircular canal-like structure was observed in some *Six1* homozygotes at E12.5, we further analyzed additional markers that are well characterized and localized along the dorsoventral axis of the otic vesicle. *Nkx5.1* is expressed early in the otic placode and its expression is restricted to the dorsolateral otic vesicle, which will give rise to the vestibular apparatus of the inner ear (Fig. 6G) (Hadrys et al., 1998; Wang et al., 1998). Inactivation of *Nkx5.1* leads to agenesis of the semicircular canals and circling behavior (Hadrys et al., 1998). In *Six1*$^{-/-}$ embryos, *Nkx5.1* expression was normal in the otic placode at E8.5 (data not shown). However in the otic vesicle, *Nkx5.1* expression was excluded from the dorsolateral region at E10.5 (arrow, Fig. 6H) and its expression shifted or expanded ventrally, including the ventral-most wall (Fig. 6G,H). *Gata3* is normally expressed throughout the otic placode and by E10.5 its expression is restricted to two regions in the otic vesicle, strongly in the dorsolateral region and weakly in the ventromedial region (Fig. 6I) (Lawoko-Kerali et al., 2002). In *Six1*$^{-/-}$ embryos, no significant difference of *Gata3* expression was detected by E9.5 (data not shown). However similar to *Nkx5.1*, *Gata3* expression in the dorsolateral region was shifted ventrally in *Six1*$^{-/-}$ otic vesicle at E10.5 (Fig. 6I,J). By contrast, its expression in the medial region was unaffected in *Six1*$^{-/-}$ otic vesicle (Fig. 6I,J). The failure to express *Nkx5.1* and *Gata3* in the dorsolateral region of the otic vesicle lacking *Six1* is unlikely to be due only to abnormal cell death, as apoptotic cells were detected throughout the lateral region at E9.5 (Fig. 3J). Although it is unclear whether the ventrolateral region of *Six1*$^{-/-}$ otic vesicle expressing *Nkx5.1* and *Gata3* will give rise to the vestibule-like structure observed in some *Six1*$^{-/-}$ embryos at E12.5, these data indicate that *Six1* regulates the establishment of regional specification of the otic vesicle.

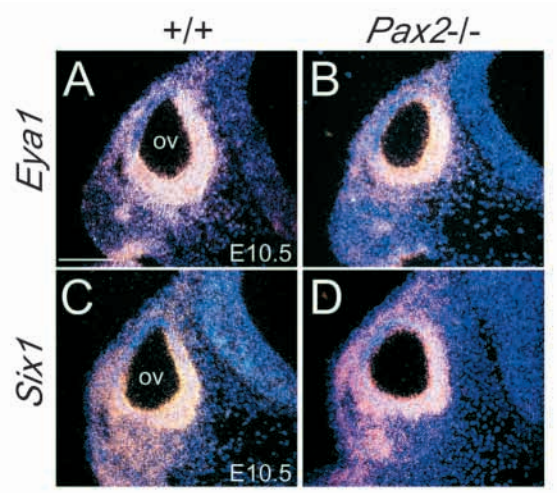

Fig. 7. *Six1* and *Eya1* expression in the otic vesicle is *Pax2* independent. (A-D) *Eya1* (A,B) and *Six1* (C,D) are expressed in the ventral otic vesicle (ov) and its derivative gVIII of wild-type embryos at E10.5 and their expression was unaffected in these structures in *Pax2*$^{-/-}$ embryos. Scale bars: 100 μm.

Six1 and *Eya1* expression in otic vesicle is *Pax2* independent

Because the *Pax2* mutant inner ear phenotype is less severe than that seen in *Eya1*$^{-/-}$ or *Six1*$^{-/-}$ mice (Torres et al., 1996), it is unclear whether *Pax* genes function in the same genetic pathway with *Eya1* and *Six1*. To further clarify their genetic relationships during early inner ear development, we next examined the expression of *Eya1* and *Six1* in *Pax2*$^{-/-}$ embryos. Surprisingly, the expression of both *Eya1* and *Six1* was unaffected in the otic vesicle and its derivative gVIII of *Pax2*$^{-/-}$ embryos (Fig. 7A-D and data not shown), indicating that *Six1* and *Eya1* expression in the otic epithelium does not require *Pax2* function. Taken together, our results suggest that *Pax2* may function independently or in parallel with *Eya1* and *Six1* during early mammalian otic development.

Eya1 and *Six1* interact during mammalian inner ear morphogenesis

As *Eya1* and *Six1* genes function in the same genetic pathway in early otic development and both gene products physically interact in vitro and in cultured cells (Buller et al., 2001), we further tested whether these two genes interact in a molecular pathway during mammalian inner ear morphogenesis by examining the inner ear gross structure of *Six1*$^{+/-}$/*Eya1*$^{+/-}$ compound heterozygotes using latex paintfilling (Table 1 and Fig. 8). At E16.5, the membranous labyrinth developed to its mature shape and the cochlea reached 1.75 turns (Morsli et al., 1998). The inner ear phenotypes in each single or double heterozygous mice were variable (Table 1). Each single heterozygote alone on 129 background revealed malformed endolymphatic duct and sac and ~18% of *Six1*$^{+/-}$ or *Eya1*$^{+/-}$ ears exhibited slightly shortened cochlea (arrowhead and arrow, Fig. 8B; data not shown). Some *Six1*$^{+/-}$ ears also revealed a small or mis-shaped saccule (asterisk, Fig. 8B). By contrast, 19 out of 40 (47.5%) *Eya1* and *Six1* compound heterozygous ears revealed more severely affected cochlea (Table 1). The severe phenotype showed a coiled but abnormal cochlea, which only completed less than 1 turn at E16.5 with abnormally shaped distal tips (insets, Fig. 8C,D). In addition, seven out of 40 (17.5%) *Eya1/Six1* double heterozygous ears showed a missing posterior ampulla and a truncation or complete absence of the posterior semicircular canal (asterisk, Fig. 8D). This defect was not seen in each single heterozygote on 129 background (Table 1). No enhancement of the defects of the endolymphatic duct and sac and the saccule was observed in the compound heterozygous inner ears (Table 1). Similar observation was obtained in C57BL/6J background (data not shown). In summary, these data suggest that *Eya1* and *Six1* genetically interact during inner ear morphogenesis and this interaction is crucial for the normal morphogenesis of the cochlea and the posterior ampulla.

We next analyzed early otic development in *Eya1/Six1* double homozygous embryos (Fig. 8E-H). At E9.5, the otic vesicle in *Eya1*$^{+/-}$/*Six1*$^{+/-}$ double heterozygotes was well formed at rhombomere 5 (r5) level and showed a restricted expression of *Six1*^{lacZ} (Fig. 8E). In the double homozygotes, the otic vesicles appeared to be formed in the correct place at r5 level but were severely hypoplastic (Fig. 8F-H). Because *Six1*^{lacZ} expression in the otic vesicle is *Eya1* dependent (Fig. 5A,B), the otic vesicle of *Eya1/Six1* double homozygous embryos lacked the *Six1*^{lacZ} expression (arrows, Fig. 8F-H). Although the neural tube of the hindbrain was significantly reduced in size in *Eya1*$^{-/-}$/*Six1*$^{-/-}$ embryos, it appeared to be patterned correctly and *Six1*^{lacZ} expression was ectopically turned on in r2, r4 and r6 (Fig. 8F-H). These data further indicate that both *Eya1* and *Six1* are not required for the initiation of otic placode morphogenesis to form the otic vesicle, but are required for the normal growth of the otic vesicle.

DISCUSSION

Six1 regulates normal growth of the otic epithelium

Inner ear development begins with the induction of the otic placode. Once the otic placode initiates to form the otic vesicle, extensive morphogenetic processes and cellular events, such as differentiation, proliferation and apoptosis, take place to ensure the formation of the highly organized structures of the adult inner ear. *Six1* expression is turned on in the invaginating otic placode at around E8.75 and in the absence of *Six1*, the otic vesicle formed without an apparent morphological alteration at E9.5. Thus, *Six1* is unlikely to be involved directly in either the induction of the otic placode or the initiation of the otic placode morphogenesis to form the otic vesicle. This conclusion was further strengthened by the fact that the otic vesicle also formed in the correct position in *Eya1/Six1* double homozygous embryos. Taken together, these findings demonstrate that both *Eya1* and *Six1* are not required for the initiation of mammalian inner ear organogenesis.

Our studies clearly demonstrate that *Six1* controls the inner ear morphogenesis by regulating the programmed cell death and proliferative growth of the otic epithelium, directly or indirectly. Secreted diffusable factors are proposed to play roles in the growth regulation of the inner ear. Of particular interest, retinoic acid (RA) and the growth factor Bmp4 have been shown to influence regional patterning and specification of the inner ear, particularly for the vestibular structures

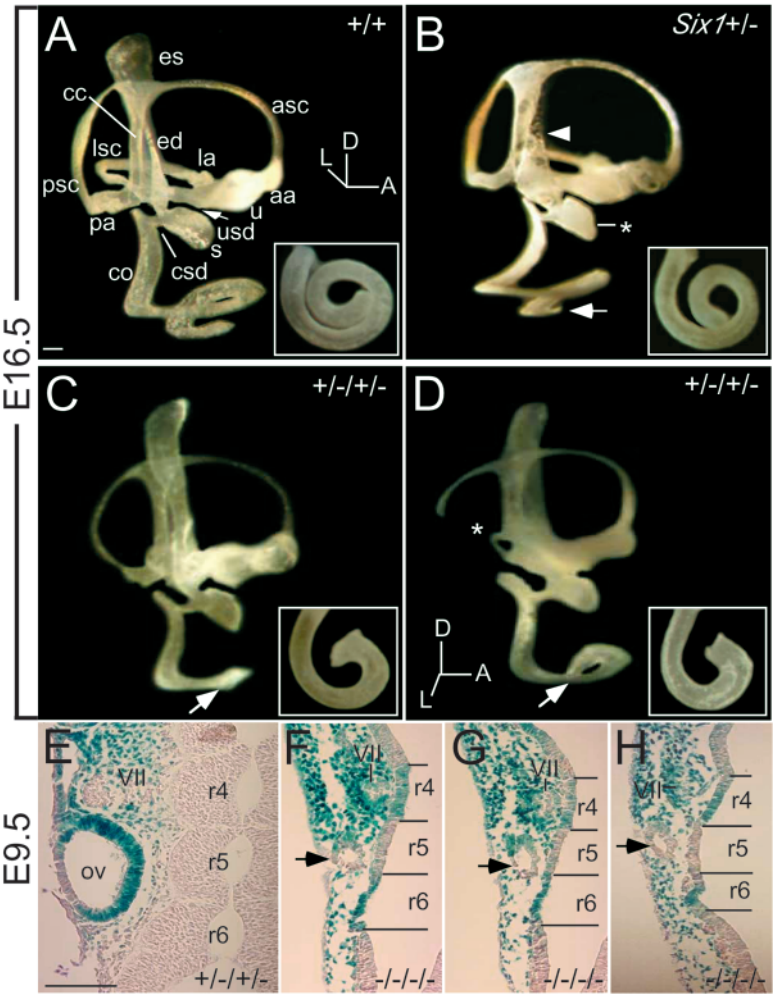

Fig. 8. Enhancement of inner ear defects in *Eya1/Six1* double mutants. (A-D) Medial (A-C) and lateral (D) views of paintfilled inner ears of wild-type (A), *Six1*$^{+/-}$ (B), and *Six1*$^{+/-}$/*Eya1*$^{+/-}$ compound heterozygous (C,D) embryos at E16.5. (A) All structures of the inner ear reached their mature shape at E16.5. The cochlea completed 1.75 turns by this stage (inset). aa, anterior ampulla; asc, anterior semicircular canal; cc, common crus; co, cochlea; csd, cochleosaccular duct; ed, endolymphatic duct; es, endolymphatic sac; la, lateral ampulla; lsc, lateral semicircular canal; pa, posterior ampulla; psc, posterior semicircular canal; s, saccule; u, utricle; usd, utriculosaccular duct. (B) A *Six1*$^{+/-}$ inner ear showing a malformed saccule (asterisk), absence of the endolymphatic sac and a truncated endolymphatic duct (arrowhead). The cochlea only completed 1.5 turns (arrow and inset). (C,D) Inner ears from *Six1*$^{+/-}$/*Eya1*$^{+/-}$ double heterozygotes showing severe cochlear defects (arrows), absence of posterior ampulla (asterisk) and truncated posterior semicircular canal. The cochlea only completed less than one turn and their distal tips were enlarged and mal-shaped (insets). (E-H) Frontal histological sections at comparable levels of X-gal-stained E9.5 embryos of *Eya1/Six1* double heterozygous (E) and double homozygous embryos (F-H). *Eya1/Six1* double heterozygote shows restricted *Six1*^{lacZ} expression in the otic vesicle (ov). In the double homozygotes, the otic vesicles appeared to be formed in the correct position but severely hypoplastic. Because *Six1*^{lacZ} is *Eya1* dependent, it is not expressed in *Eya1*$^{-/-}$/*Six1*$^{-/-}$ otic vesicle (arrows) but is ectopically turned on in rhombomeres 2, 4 and 6 (r4 and r6). Scale bars: 100 μm.

(Chang et al., 1999; Dupe et al., 1999; Gerlach et al., 2000; Niederreither et al., 2000; Pasqualetti et al., 2001; Merlo et al., 2002). Interestingly, the inner ears of *Six1*$^{-/-}$ embryos bear some similarities to the phenotype displayed by mice exposed to excess all-trans RA (at-RA) or isotretinoin (13*cis*-RA), or mice lacking *Raldh2*, a gene that catalyzes RA formation (Burk and Willhite, 1992; Niederreither et al., 2000). In the otic vesicle of both *Six1*$^{-/-}$ and *Raldh2*$^{-/-}$ embryos, *Nkx5.1* expression is expanded ventrally (Niederreither et al., 2000). RA is also able to rescue the *Hoxa1*$^{-/-}$ inner ear phenotype, including vestibular malformation and a lack of cochlear duct outgrowth (Pasqualetti et al., 2001). Recently, Bmp4 has been suggested to function together with RA through the same pathway or intersecting pathways, as RA represses *Bmp4* transcription in otocyst cells (Thompson et al., 2003). However, it is unclear how exactly the RA and Bmp4 signaling controls the patterning of the inner ear. In the present study, we found that the maintenance of *Bmp4* expression in the otic vesicle requires *Six1* function. Therefore, it is possible that *Six1* regulates normal growth of the otic vesicle through the RA-Bmp4-signaling pathway. This could explain why the morphogenetic defects in *Six1*$^{-/-}$ mice are not restricted to the ventral inner ear but extend to the dorsal inner ear where *Six1* is not expressed. Thus, the disruption of Six1 exerts some indirect, nonautomous effects on developing inner ear structures. Such long-distance influence may also be regulated by the expression of *Six1* in the periotic mesenchyme. It will be interesting to test whether RA can rescue the *Six1*$^{-/-}$ inner ear phenotype.

The role of *Six1* in the specification of neuroblast cells

As the otic placode invaginates, a population of otic epithelial cells near the center of the otic cup and ventral otic vesicle emigrates into the underlying mesoderm. These cells are neuroblasts for the vestibuloacoustic ganglion (gVIII) and they are the first cell lineage specified within the otic epithelium before leaving the otic epithelium. The basic helix-loop-helix (bHLH) transcription factors neurogenin 1 (Ngn1) and Neurod1 have been shown to be essential for the formation of gVIII (Ma et al., 1998; Liu et al., 2000; Kim et al., 2001). *Ngn1* was proposed to play a role for the determination of neuroblast precursor fate (Ma et al., 1998), while *Neurod1* is required for the delamination of neuroblasts and for their survival during differentiation process (Liu et al., 2000; Kim et al., 2001). Recently, the transcription coactivator Eya1 has been shown to be required for the formation of the gVIII, as this structure failed to form in *Eya1*$^{-/-}$ mice (Xu et al., 1999a). The zinc-finger protein Gata3 is expressed in the neuroblasts and also plays a role in the formation of gVIII (Karis et al., 2001).

Growth factors Fgf3 and Fgf10 and their receptor Fgfr2 IIIb also play a role in the formation of the gVIII, as mutations in each of these genes led to severe hypomorphic development of the gVIII (Mansour et al., 1993; Pirvola et al., 2000). However, the molecular mechanisms controlling the specification of neuroblast cell fate are currently not well understood. In the present work, we found that *Six1* is expressed in the ventral otic epithelium within which the neuroblast precursors are specified and in the gVIII. In the absence of *Six1*, the gVIII failed to form, similar to that observed in *Eya1*$^{-/-}$ embryos. Thus, *Six1* is likely to play a direct role in the determination of neuroblast cell fate and this cell lineage may not be specified in the absence of *Six1*. This hypothesis was further supported by loss of specific marker expression, including *Fgf3* and *Fgf10* in the neuroblasts and gVIII of *Six1*$^{-/-}$ embryos (Fig. 6 and data not shown).

As both *Eya1* and *Six1* are required for the activation of *Fgf3* expression in the otic epithelium and both proteins physically interact in vitro and in cultured cells (Buller et al., 2001), it is possible that *Fgf3* is a common downstream target for both *Eya1* and *Six1*. Based on these observations, we propose that both *Eya1* and *Six1* control the initial selection of neuroblast precursors by regulating the expression of *Ngn1*, *Neurod1* and *Fgf3*, directly or indirectly. This hypothesis will be strengthened if *Ngn1* and *Neurod1* expression in the otic epithelium and neuroblasts also requires both *Eya1* and *Six1* function. Expression studies of *Ngn1* and *Neurod1* in both *Eya1* and *Six1* mutants are under way in our laboratory.

The role of *Six1* in the specification of sensory regions

The commitment of the otocyst to form prospective vestibular and auditory sensory areas is controlled by patterning genes. However, it is unclear exactly how these genes are involved in this complicated process. Our studies show that *Six1* is expressed in the middle and ventral otic vesicle and all middle and ventral derivatives failed to form in the absence of *Six1*. To analyze the molecular defects in *Six1*$^{-/-}$ otic vesicle, we examined the expression of ventromedial otic markers, including *Pax2*, *Pax8*, the zinc-finger gene *Sall1* and dachshund 1 (*Dach1*), in *Six1*$^{-/-}$ embryos and failed to detect obvious changes in their expression between wild-type and *Six1*$^{-/-}$ embryos. In addition, *Gata3* expression in the ventromedial region was also unaffected in *Six1*$^{-/-}$ embryos. Further studies are required to establish the molecular mechanisms by which *Six1* acts to regulate the patterning of this region in the inner ear.

Interestingly, in addition to the loss of ventral cell fates, the absence of *Six1* impacts the positioning of dorsolateral markers, including *Nkx5.1* and *Gata3*. Although *Nkx5.1* appears to be necessary for the correct expression of *Bmp4* in the otocyst and for regional control of apoptosis and *Bmp4* was suggested to have a role in the specification of sensory organ formation (Oh et al., 1996; Morsli et al., 1998; Cole et al., 2000; Merlo et al., 2002), it is unclear how these genes function together to control the morphogenesis of the sensory system. We found that *Six1* is expressed in all sensory regions of the inner ear and *Six1*$^{-/-}$ mice lacked all sensory organ formation. The expression domain of *Bmp4*, which marks the sensory anlage of the posterior crista was lost and its expression level in the other sensory anlagen was also largely reduced in *Six1*$^{-/-}$ embryos. Coincidentally, *Eya1* and *Six1* interaction critically affects the morphogenesis of the posterior ampulla and *Bmp4* expression was also lost in *Eya1*$^{-/-}$ embryos at E10.5 (data not shown). Therefore, it is likely that both *Eya1* and *Six1* regulate the expression of *Bmp4* the dosage of which is crucial for the morphogenesis of the sensory organs, particularly for the posterior ampulla. Nonetheless, our results indicate that *Six1* is probably an early regulator for the specification of all sensory organs of the inner ear.

The regulatory relationship between Pax, Eya and Six genes during mammalian inner ear morphogenesis

In the ear, both *Eya1* and *Six1* are co-expressed during mammalian auditory system development and their mutant mice had similar defects in all three parts of the ear (Xu et al., 1999a). Our studies have clearly demonstrated that the *Drosophila* Eya-Six regulatory cassette is evolutionarily conserved during mammalian inner ear development.

In *Drosophila* eye imaginal disc, the fly *Pax6* gene *ey* has been shown to function upstream of both *eya* and *so* (Halder et al., 1998). In mammalian inner ear, *Pax2* expression overlaps with *Eya1* and *Six1* in the medial otic vesicle and the inner ear phenotype in *Pax2*$^{-/-}$ mice is less severe than that seen in *Eya1*$^{-/-}$ or *Six1*$^{-/-}$ mice (Torres et al., 1996). *Pax8*, a paralog of *Pax2*, is also expressed in the otic placode (Pfeffer et al., 1998). Although *Pax8* mutants do not exhibit an otic phenotype (Mansouri et al., 1998), *Pax2* and *Pax8* may function redundantly during early otic morphogenesis with *Pax2* alone executing later functions. This could explain why the *Pax2* mutant phenotype appears to occur slightly later. If Pax2 and Pax8 function redundantly in early otic development and a crucial threshold of Pax2/Pax8 protein expression in otic epithelium regulates *Eya1* and *Six1* expression, *Eya1* and *Six1* expression should be reduced or lost in *Pax2/Pax8* double homozygotes. We are currently testing this hypothesis by generating *Pax2/Pax8* compound mutants in C3H/He background, as *Pax2/Pax8* compound heterozygous females in either 129 or C57BL/6J strain had a blind-ending vagina, similar to the recent observation by Bouchard et al. (Bouchard et al., 2002). Alternatively, *Pax2* and *Pax8* could function in parallel or independently of *Eya1* and *Six1* in early otic morphogenesis, as *Eya1* and *Six1* expression was unaffected in *Pax2*$^{-/-}$ otic vesicle. Evidence obtained from the analysis of the cochlea phenotype in *Eya1/Pax2* compound heterozygous mice suggests that *Eya1* and *Pax2* may interact during cochlear development, because the cochlea phenotype is enhanced in *Eya1/Pax2* compound heterozygotes than in each single heterozygote (data not shown). It should also be noted that our recent studies indicate that the genetic and regulatory relationship between Pax, Eya and Six genes varies between different organs during mammalian development (Xu et al., 2003). Probably, the Pax, Eya and Six genes function in the same or parallel pathway but with different combinations of regulatory relations in different organs. Detailed examination of inner ears in *Pax2/Pax8*, *Pax2*/*Eya1*, *Pax2/Six1* or *Eya1/Six1/Pax2* compound knockouts will enhance our understanding on the possible molecular and genetic interactions between these transcription factors during early mammalian inner ear morphogenesis.

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