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Role of hindbrain in inner ear morphogenesis: Analysis of *Noggin* knockout mice

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Abstract

Signaling from rhombomeres 5 and 6 of the hindbrain is thought to be important for inner ear patterning. In *Noggin* −/− embryos, the gross anatomy of the inner ear is distorted and malformed, with cochlear duct outgrowth and coiling most affected. We attributed these defects to a caudal shift of the rhombomeres caused by the shortened body axis and the kink in the neural tube. To test the hypothesis that a caudal shift of the rhombomeres affects inner ear development, we surgically generated chicken embryos in which rhombomeres 5 and 6 were similarly shifted relative to the position of the inner ears, as in *Noggin* mutants. All chicken embryos with shifted rhombomeres showed defects in cochlear duct formation indicating that signaling from rhombomeres 5 and 6 is important for cochlear duct patterning in both chicken and mice. In addition, the size of the otic capsule is increased in *Noggin* −/− mutants, which most likely is due to unopposed BMP signaling for chondrogenesis in the peri-otic mesenchyme.
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Keywords: Noggin; BMP; Inner ear; Rhombomere; Hindbrain; Chondrogenesis

Introduction

The formation of the structurally complex vertebrate inner ear requires instructive signaling from surrounding tissues such as the hindbrain, mesenchyme, neural crest, and endoderm. Among these tissues, the hindbrain, in particular, has received the most attention because of existing hindbrain mutants with inner ear defects such as the *Kreisler* and *Hoxa1* knockout mice (for review, see Kiernan et al., 2002). More detailed analyses of these and other mutants indicate that rhombomeres 5 and 6 (r5 and r6) of the hindbrain, which are located immediately adjacent to the developing inner ear, are important for its patterning.

Noggin is a secreted polypeptide first isolated from the Spemann's organizer in *Xenopus* (Smith and Harland, 1992). It functions as an antagonist of BMP pathways by binding to BMPs with high affinity, in particular BMP2 and BMP4 and preventing them from activating their receptors (Holley et al., 1996; Zimmerman et al., 1996). Gain of function studies in frogs and fish implicated a role for Noggin in dorsalizing the embryo with respect to specification of neural and mesodermal fates (for review, see De Robertis and Kuroda, 2004). However, analyses of *Noggin* knockout mouse embryos indicate that Noggin is not required for neural specification or head formation in mammals but is required for proper differentiation of the caudal neural tube and formation of somites and joints (Brunet et al., 1998; McMahon et al., 1998). In *Noggin* mutants, the neural tube forms normally but becomes kinked by 9.5 days post coitum (dpc), and the body axis is shortened. Some mutant embryos also display exencephaly. Beside the kinks and exencephaly, gene expression analyses indicate that the part of the neural tube rostral to the forelimbs is fairly normal, whereas the differentiation of caudal neural tube and somites is severely affected in *Noggin* mutants (McMahon et al., 1998).

Mutations of the *NOGGIN* gene in humans are associated with several autosomal dominant disorders that are characterized

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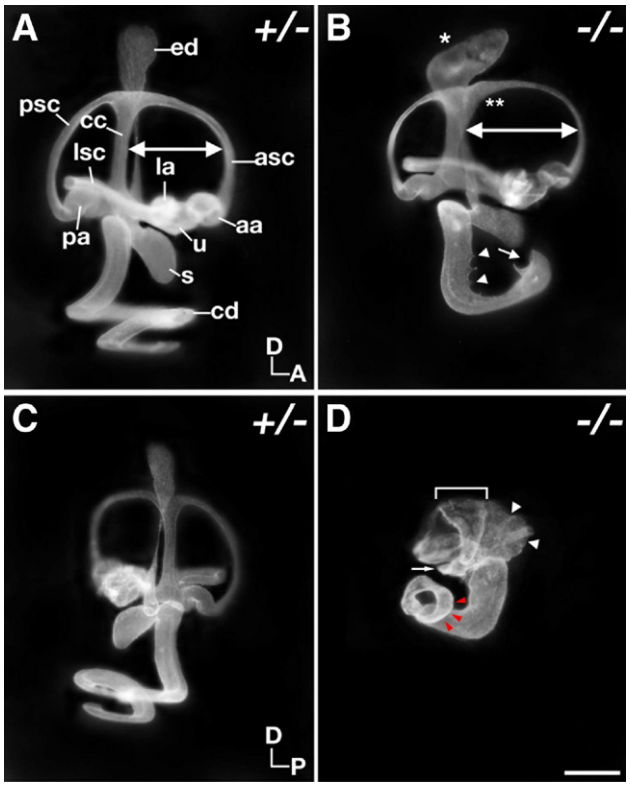

Fig. 1. Inner ear phenotypes of *Noggin* −/− mutants at 16.5 dpc. Lateral (A, B) and medial (C, D) views of paint-filled right inner ears from *Noggin* +/− (A, C) and −/− mutants (B, D). The *Noggin* mutant ear in (B) shows a malformed endolymphatic duct (asterisk), a widened and slanted common crus (double asterisks), an extended anterior canal (double headed arrow), and a malformed cochlear duct with protrusions (arrowheads) and mis-coiling towards the top (arrow). The *Noggin* mutant ear in (D) is smaller in size with a non-resorbed posterior canal (white arrowheads) and malformed saccule (arrow). The cochlear duct is coiling medially in the wrong direction (red arrowheads). A bracket marks the width of the endolymphatic duct at the top. Abbreviations: aa, anterior ampulla; asc, anterior semicircular canal; cd, cochlear duct; cc, common crus; ed, endolymphatic duct; la, lateral ampulla; lsc, lateral semicircular canal; pa, posterior ampulla; psc, posterior semicircular canal; s, saccule; u, utricle. Orientations: A, anterior; D, dorsal; P, posterior. Scale bar = 500 μm.

by skeletal and joint defects such as proximal symphalangism (SYM1) and multiple-synostoses syndrome (SYNS1) (Gong et al., 1999; Takahashi et al., 2001). Conductive hearing loss due to fixation of the stapes to the temporal bone is also a manifestation of *NOGGIN* mutations (Brown et al., 2002; Gong et al., 1999; Weekamp et al., 2005).

In *Noggin* −/− mouse mutants, the eyes and ears are also malformed (McMahon et al., 1998). Since BMPs have been implicated in inner ear development (Chang et al., 1999, 2004; Gerlach et al., 2000), we analyzed the inner ears of *Noggin* knockout mice as a potential animal model for gain of BMP functions. Our results show that the gross anatomical defects observed in the inner ears of *Noggin* mutants are largely due to misalignment of the otocysts with the hindbrain during early development rather than a mis-regulation of BMP levels within the inner ear. However, the increase in otic capsule size observed in the *Noggin* mutants is most likely due to a net gain of BMP functions.

Materials and methods

Mice, paint-fills, in situ hybridization and β-gal histochemistry

Noggin homozygous mice in C57BL/6 or a mixed C57BL/6 and FVB background where indicated were generated and genotyped as described (Brunet et al., 1998). Gross anatomical analyses by injecting 0.1% alkyd white paint solution in methyl salicylate to the cavity of the inner ear and in situ hybridization experiments were performed as described (Morsli et al., 1998). A total of 8 embryos between 12 and 13.5 dpc and 15 embryos between 15.5 and 17.5 dpc were processed for paint-fill analyses. A total of 32 *Noggin* homozygous embryos were used for gene expression analyses: 26 between 8.5 and 10.5 dpc, 5 between 11.5 and 12.5 dpc, and 9 between 14.5 and 16.5 dpc. RNA probes for *Aggrecan* (Watanabe et al., 1995), *Bmp2* (Lyons et al., 1989), and *Bmp4* (Morsli et al., 1998) were generated as described. A Pst1–ApaI fragment from nucleotides 1142 to 1895 of *Krox20* cDNA (NM_010118) including the zinc-finger region was used to generate RNA probes for *Krox20* (gift of David Wilkinson). *lacZ* plasmid for generation of RNA probes was a gift from David Lin at Cornell University. RNA probes for *Hoxb1* were generated from nucleotides 71 to 1017 of the *Hoxb1* cDNA (NM_008266). For the detection of *Pax8* expression, RNA probes were generated from two cDNA regions, a 184 base pair fragment described by Planchov et al. (1990) and a 674 base pair fragment from nucleotides 595 to 1269 (NM_011040).

β-Galactosidase (β-gal) histochemistry on Noggin +/− embryos at 9.5 dpc was performed by fixing embryos with 1% formaldehyde/0.2% glutaraldehyde for 30 min, before processing for β-gal staining as described (Epstein et al., 2000). Embryos at 11.5 to 16.5 dpc were first fixed with 2% paraformaldehyde overnight and then processed for cryosectioning and β-gal histochemistry.

Chicken, surgical procedures

Fertilized eggs (CBT farms, MD) were incubated at 37 °C in a humidified chamber. For rhombomere transplantation procedures, embryos at embryonic day 1.5 (E1.5) equivalent to 12–15 somite stages (ss) or Hamburger Hamilton stage 10–12 (HH 10–12) were used (Hamburger and Hamilton, 1951). A number of surgical permutations were performed (see Results section) before deciding on the optimal condition, which was performed by removing a segment of the hindbrain between r3 and r6 using a micro-surgical blade. Then, the isolated hindbrain segment was transplanted to an age-matched host embryo with r5 to r7 removed for rhombomere-shifts or r3 to r6 removed for controls. The operated embryos were further incubated and subsequently harvested at E2.5 for gene expression analyses using whole mount in situ hybridization, or harvested at E9 for anatomical analyses using the paint fill technique (Bissonnette and Fekete, 1996).

Results

Inner ear phenotype of Noggin knockout mice

The anatomy of *Noggin* mutant inner ears was visualized by injecting 0.1% alkyd white paint solution to the cavity of the inner ear (Fig. 1). A total of 15 homozygous mutant embryos

Table 1
Frequency of malformed inner ear structures in *Noggin* −/− mutants

	Affected inner ear structures				
	Endolymphatic duct	Common crus	Semicircular canals	Saccule	Cochlear duct
# of affected specimens/ total	12/12	13/14	11/14	9/15	15/15