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Catweasel mice: A novel role for *Six1* in sensory patch development and a model for branchio-oto-renal syndrome

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ABSTRACT

Large-scale mouse mutagenesis initiatives have provided new mouse mutants that are useful models of human deafness and vestibular dysfunction. Catweasel is a novel *N*-ethyl-*N*-nitrosourea (ENU)-induced mutation. Heterozygous catweasel mutant mice exhibit mild headtossing associated with a posterior crista defect. We mapped the catweasel mutation to a critical region of 13 Mb on chromosome 12 containing the *Six1*, *-4* and *-6* genes. We identified a basepair substitution in exon 1 of the *Six1* gene that changes a conserved glutamic acid (E) at position 121 to a glycine (G) in the Six1 homeodomain. *Cwe*/*Cwe* animals lack Preyer and righting reflexes, display severe headshaking and have severely truncated cochlea and semicircular canals. *Cwe*/*Cwe* animals had very few hair cells in the utricle, but their ampullae and cochlea were devoid of any hair cells. *Bmp4*, *Jag1* and Sox2 expression were largely absent at early stages of sensory development and *NeuroD* expression was reduced in the developing vestibulo-acoustic ganglion. Lastly we show that *Six1* genetically interacts with *Jag1*. We propose that the catweasel phenotype is due to a hypomorphic mutation in *Six1* and that catweasel mice are a suitable model for branchio-oto-renal syndrome. In addition *Six1* has a pivotal role in early sensory patch development and may act in the same genetic pathway as *Jag1*.

Introduction

Over 300 human syndromes have been described where deafness and/or vestibular malfunction are present (Tekin et al., 2001) and many of these syndromes have severe malformations of the hearing apparatus due to abnormal inner ear development. Large-scale ENU and other mutagenesis programmes have provided the field with new mouse mutants with deafness and/or vestibular dysfunction. This has allowed us and others to identify novel candidate genes for human deafness and some of these provide good models for human diseases (Kiernan et al., 2001, 2005; Bosman et al., 2005; Vrijens et al., 2006).

Branchio-oto-renal syndrome (OMIM 113650; http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=113650) is an autosomal dominant developmental disorder of kidney and urinary tract malformations and hearing impairment (Melnick et al., 1976). In humans, mutations in *EYA1* and its interacting partners *SIX1* and *SIX5* have been identified as causing BOR syndrome (Abdelhak et al., 1997; Ruf et al., 2004; Hoskins et al., 2007). This syndrome has a wide intrafamilial variability and reduced penetrance (Kumar et al., 1999). A closely related disorder is branchio-oto (BO) syndrome, where patients suffer from branchial defects and deafness without renal abnormalities (OMIM 602588), but might be a milder variant of BOR syndrome. Deletion of the *Six1* or *Eya1* genes in mice has confirmed the important role of these genes during the development of inner ear and other organs affected in BOR syndrome. Mice carrying a hypomorphic *Eya1* mutation have inner ear and other malformations that are reminiscent of those found in patients with BOR syndrome (Johnson et al., 1999). Complete loss of *Eya1* and *Six1* leads to an arrest in inner ear development at otocyst stage due to a failure of dorso-ventral (D-V) axis determination (Xu et al., 1999; Zheng et al., 2003). In addition to this early role, *Six1* and *Eya1* are both expressed during later stages of inner ear morphogenesis mainly in the developing sensory epithelium and a role for *Eya1* during sensory patch specification has been proposed (Zheng et al., 2003; Ozaki et al., 2004; Zou et al., 2006). The role of *Six1* during later stages of inner ear morphogenesis and development of the sensory patch remains to be elucidated.

Here we describe catweasel (*Cwe*), a novel ENU-induced mutation that causes mild headbobbing in heterozygous (*Cwe*/+) mice. These mice lack the eminentium cruciatum in the posterior crista and have extra inner hair cells in the organ of Corti. Catweasel maps to a 13 Mb region of chromosome 12 and we identified a point

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mutation in the gene encoding the Six-type homeobox protein Six1. Homozygous (*Cwe*/*Cwe*) mice are viable, but have kidney defects, severe vestibular abnormalities and are deaf, due to defects in both the inner and middle ear. These mice lack most sensory hair cells, and we show that sensory patch development is affected as early as E10.5. Finally we show that *Six1* interacts genetically with the Notch ligand *Jag1*. We propose that *Six1* has a pivotal role in early sensory patch development and may act in the same genetic pathway as *Jag1*. In addition, catweasel mice are a good model to study the inner ear and kidney abnormalities found in branchio-oto-renal (BOR) syndrome.

Materials and methods

Mice and behavioural analysis

Animal husbandry and experiments were carried out in accordance to UK Home Office regulations and with permission of the government of Oberbayern (Germany). The catweasel mutation arose from a large-scale mutagenesis screen (Hrabé de Angelis et al., 2000). Male C3HeB/FeJ mice were injected with three doses of 80 mg/kg *N*-ethyl-*N*-nitrosourea (ENU) at weekly intervals, allowed to recover and mated with uninjected C3HeB/FeJ females. F1 offspring were screened for a variety of dominantly inherited defects including deafness and vestibular defects. A custom built click box was held above the mouse and a calibrated 20 kHz tone burst at an intensity of 90 dB SPL was delivered and the presence of an ear flick response (Preyer reflex) was recorded. Other behavioural testing (rotarod, swim test, tail suspension) was performed as described (http://www.eumorphia.org/EMPReSS/servlet/EMPReSS.Frameset). The catweasel (*Cwe*, ABE4) founder was discovered because of its mild headshaking behaviour. For all analysis, unless otherwise described, the mutants used were distinguished by their clear headbobbing behaviour, and the mice were studied on their original C3HeB/FeJ genetic background.

Genetic mapping and genotying

Cwe/+ animals on a C3HeB/FeJ genetic background were outcrossed to C57BL/6JIco mice. Affected *Cwe*/+ F1 offspring were backcrossed to wild type (+/+) C3HeB/FeJ animals. The DNA from 30 backcross offspring exhibiting severe headshaking behaviour was used in a genome scan to link the behavioural trait with a chromosome. A panel of 57 markers spanning the autosomes was used to detect polymorphisms between C3HeB/FeJ and C57BL/6JIco mice (Suppl. Table 1). Fine mapping was carried out using *Cwe*/+ mutants with confirmed abnormal posterior crista ($n=38$) with additional markers on chromosome 12 (Suppl. Table 2). To genotype catweasel mice, *Six1* exon 1 was amplified by PCR using FW 5′-CACCTGCACAAGAACGAGAG-3′ and RV 5′-TTCGACTCAGACCAGCTTCA-3′ primers and sequenced with internal primer FW2 5′-ACTTCCGCGAGCTCTACAAG-3′.

Phenotype analysis

P21 mice were sacrificed by cervical dislocation. Scanning electron microscopy was performed as described (Bosman et al., 2005). Middle ear ossicles were dissected out in PBS and photographed. For inner ear examination heads were bisected, the brain removed and the skull was fixed in Bodian's fixative (75% ethanol, 5% acetic acid, 5% formaldehyde in water) overnight, washed in water and 70% ethanol and treated with 3% potassium hydroxide in water at room temperature for 1 week. After further dissection of the inner ear, the inner ears were cleared overnight in a mixture of glycerol: 70% ethanol: benzol (2:2:1) and photographed in 70% ethanol: benzol (1:1). Paintfilling of the inner ear was performed as described (Bissonnette and Fekete, 1996; Kiernan, 2006).

In situ hybridisation and immunohistochemistry

Wildtype, *Cwe*/+, *Cwe*/*Cwe*, *Htu*+ and *Htu*/+; *Cwe*/+ embryos from timed matings were dissected in ice-cold PBS at E10.5 to E16.5, with E0.5 at noon the day the vaginal plug was found. For the marker analysis on sections 3 embryos of each genotype were used, for whole mount in situ hybrisation 4 embryos of each genotype were used. For whole mount in situ hybridisation embryos were fixed overnight at 4 °C in 4% paraformaldehyde in PBS and processed as described (Albrecht et al., 1997). For in situ hybridisation and immunohistochemistry on sections samples were fixed overnight at 4 °C in 10% neutral-buffered formalin, embedded in paraffin and cut into 8 μm sections and the Ventana Discovery system (Ventana Medical Systems, Inc Illkirch, France) was used according to the manufacturer's instructions. Plasmids containing cDNA of *Bmp4* (Jones et al., 1991), *Jag1* (Mitsiadis et al., 1997) and *Six1* (gift Dr. N. Bobola) and antibodies against Sox2 (Abcam, Cambridge, UK, cat. no. ab15830), Calretinin (Chemicon international, Millipore, Hampshire, UK, cat. no. AB5054), Myo7A (Proteus) and Jag1 (Santa Cruz, Heidelberg, Germany, cat. no. sc-6011) were used. The *NeuroD* in situ hybridisation probe was generated by RT-PCR on cDNA from wildtype E10.5 embryos (primer sequences: NeuroD-FW-T3 5′-AATTAACCCTCACTAAAGGGAG-gttctcaggacgaggaacacgaggc-3′ and NeuroD-RV-T7 5′AATACGACTCAC-TATAGGGAGgcagcggcaccggaagagaagat-3′ followed by in vitro transcription using T7 polymerase to generate the antisense probe.

Results

Catweasel is a dominant mutation causing headbobbing due to a posterior crista defect

The catweasel (*Cwe*) mutation arose from a large-scale mutagenesis screen (Hrabé de Angelis et al., 2000). The catweasel founder was discovered because of its mild headshaking behaviour. Crossing the founder with wildtype C3HeB/FeJ mice showed that this phenotype had a dominant inheritance. *Cwe*/+ animals showed normal reaching, contact righting, and Preyer reflexes, normal swimming and spent normal time on the elevated platform and rotarod (data not shown). Whole skeletal preparations and analysis of the cleared adult inner ear and dissected middle ear revealed no obvious gross morphological differences between *Cwe*/+ mutants and controls (data not shown).

Scanning electron microscopy of the vestibular sensory epithelia showed no obvious abnormalities in the maculae, anterior and lateral cristae of *Cwe*/+ mice (data not shown). The posterior crista of wildtype mice normally has a non-sensory ridge running in the middle of the sensory patch, called the eminentium cruciatum (Desai et al., 2005; Fig. 1A). In the posterior cristae of *Cwe*/+ animals this eminentium cruciatum was missing ($n=11$; Fig. 1B). There was no obvious difference in the overall size of the sensory region between *Cwe*/+ animals and controls, but the two ends of the sensory patch were more rounded in shape in *Cwe*/+ mice than in controls. Three animals scored as *Cwe*/+ by their headbobbing had an incomplete eminentium cruciatum, indicating that there might be a reduced penetrance for the posterior crista defect in *Cwe*/+ mice.

Scanning electron microscopic analysis of the organ of Corti showed that adult wildtype mice had a normal pattern of one row of inner hair cells (IHC) and 3 rows of outer hair cells (OHC). These hair cells all had well-developed stereocilia bundles (Fig. 1C). In *Cwe*/+ animals, hair cells developed normally and stereocilia were well-developed showing a normal staircase arrangement. However, we observed additional IHCs along the length of the cochlear duct (Fig. 1D). Counts of the ectopic second row of inner hair cells showed significantly more additional inner hair cells in *Cwe*/+ mutants than in wild type littermates in all turns of the cochlea, but especially in the apical turn (Fig. 1E, *Cwe*/+, $n=5$ and +/+, $n=5$).