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Loss of *Sox9* in the periotic mesenchyme affects mesenchymal expansion and differentiation, and epithelial morphogenesis during cochlea development in the mouse

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ABSTRACT

Sox9 encodes an HMG-domain transcription factor that is critically required in numerous developmental processes such as chondrogenesis and otic placode formation. Here, we show that *Sox9* is expressed in the mesenchyme surrounding the developing cochlea in the mouse suggesting that Sox9 may also control development of the otic fibrocyte compartment and the surrounding otic capsule. Tissue-specific inactivation of *Sox9* in the periotic mesenchyme using a *Tbx18*^{Cre} mouse line results in arrest of early chondrogenesis and consequently, in a lack of cochlear otic capsule formation. Furthermore, loss of *Sox9* severely compromises expansion, differentiation and remodeling of the otic fibrocyte compartment. Early cell proliferation defects in the entire periotic mesenchyme of *Sox9*-deficient inner ears suggest a cell-autonomous function of Sox9 for the development of the inner mesenchymal compartment. Abnormal cochlear duct morphogenesis in *Sox9* mutants including disruption of the coiling process is tightly associated with the onset of mesenchymal defects whereas the absence of major differentiation defects in the otic epithelium suggests that Sox9-dependent mesenchymal signals primarily control epithelial morphogenesis.

Introduction

The mammalian inner ear contains the sensory organs for hearing and balance; the cochlea that perceives sound, and the vestibulum, with utricle and saccule and semicircular canals that measures torsion and linear acceleration. Unifying feature is the presence of specialized sensory cells, the hair cells that transform mechanical deflection into electrical signals, and forward them to associated neurons. Hair cells are arranged in a continuous epithelial cell layer interspersed with supporting cells that is surrounded by a layer of otic fibrocytes (termed inner compartment) and a protecting outer bony capsule (reviewed in Raphael and Altschuler (2003)). Otic fibrocytes do not only serve as a mechanical link between the epithelial cell compartment and the otic capsule but they are also critically involved in the sensory process by maintaining the ionic homoeostasis required for hair cell depolarization (reviewed in Wangemann (2006)).

Epithelial and mesenchymal compartments of the inner ear arise from distinct cell lineages during embryonic development. In the mouse, a specialized region of the surface ectoderm adjacent to the posterior hindbrain, the otic placode, starts to invaginate at E8.5 and detaches to form a vesicle, the otocyst, around E10.0. Subsequent epithelial morphogenesis generates the complex three-dimensional (3-D) architecture of the vestibulum and the coiled cochlea. Cells of the otocyst proliferate, migrate and differentiate to give rise to the neurons of the cochleovestibular ganglion and the epithelial sensory and non-sensory cells of the membraneous labyrinth (Kiernan et al., 2002; Barald and Kelley, 2004, and references therein). Mesenchymal cells of cranial paraxial mesodermal origin start to condense around the otocyst at E10.0. The cells in direct proximity to the otic epithelium will differentiate into otic fibrocytes, whereas the outer compartment of mesenchymal cells will undergo endochondral ossification to form the bony capsule that envelopes the membranous labyrinth (Sher, 1971; McPhee and Van de Water, 1985; Cohen-Salmon et al., 2000).

Tissue recombination and explant culture experiments in mice and birds suggest that the regular development of epithelial and mesenchymal compartments of the inner ear relies on intense reciprocal tissue interactions (McPhee and Van de Water, 1986; Swanson et al., 1990). In particular, factors inducing and regulating chondrogenesis, including Fibroblast growth factors (Fgfs) 2,3 and 9, Transforming growth factor (Tgf)ß1 and Bone morphogenetic

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protein 4 (Bmp4) emanate from the otic epithelium (Frenz and Van De Water, 1991; Frenz et al., 1994; Frenz and Liu, 1998; Liu et al., 2003; Pirvola et al., 2004). Conversely, the periotic mesenchyme is a source of signals that induce otic placode formation (Barald and Kelley, 2004), and control patterning and differentiation of the sensory epithelium (Montcouquiol and Kelley, 2003; Doetzlhofer et al., 2004). Experimental removal of the periotic mesenchyme decreases the morphogenetic potential of the epithelium indicating that cellular reorganizations underlying the cochlear coiling mechanism require an interaction with the surrounding mesenchyme (Miura et al., 2004). However, the *in vivo* relevance of these findings and the nature of signaling factors and transcriptional circuits that mediate and interpret tissue interactions during inner ear development have remained elusive. Genetic analyses have recently identified the POU domain, class 3, transcription factor 4 (Pou3f4) and the T-box 1 protein (Tbx1) as potential regulators of these processes. Loss of *Pou3f4* in the periotic mesenchyme results in mild defects of cochlear coiling morphogenesis (Minowa et al., 1999; Phippard et al., 1999). In contrast, conditional inactivation of *Tbx1*, a T-box transcription factor implicated in the etiology of DiGeorge syndrome, from the periotic mesenchyme, results in a rudimentary cochlea with severely compromised coiling and disrupted differentiation of the Organ of Corti. These epithelial defects were interpreted to be secondary to the disruption of pericochlear capsule formation by decreased mesenchymal proliferation (Xu et al., 2007).

Sox9 encodes a transcription factor with a conserved SRY-related high mobility group (HMG) DNA binding domain (Foster et al., 1994; Wagner et al., 1994). *Sox9* is a critical regulator of chondrogenesis but a requirement in the development of many other organs has also been reported (Akiyama et al., 2002; for summary Kist (2009)). Homozygous loss of *Sox9* leads to early embryonic lethality (Akiyama et al., 2004). Heterozygous mice die perinatally and serve as a model for campomelic dysplasia, a human skeletal malformation syndrome that is frequently associated with conductive and sensorineural hearing loss (Tokita et al., 1979; Houston et al., 1983; Foster et al., 1994; Wagner et al., 1994; Bi et al., 2001; Mansour et al., 2002).

In fact, studies in *Xenopus* and zebrafish have previously provided evidence for an additional primary function of *Sox9* in inner ear development. Morpholino antisense oligonucleotide-mediated depletion of Sox9 protein in *Xenopus* embryos results in loss of early otic markers and failure of otic vesicle development. Expression of an inducible dominant negative version of *Sox9* blocked otic development during gastrulation, but had little effect on later inner ear development, indicating that *Sox9* is required for otic placode specification, but not for subsequent processes in otic development (Saint-Germain et al., 2004). Furthermore, loss of *Sox9*a and *Sox9*b function in zebrafish results in the absence or severe reduction of the otic vesicle (Yan et al., 2005), whereas over-expression of *Sox9* in *Xenopus* causes the formation of enlarged or ectopic otic vesicles (Taylor and Labonne, 2005).

Tissue-specific *Sox9* inactivation in the early otic epithelium recently disproved a role in otic placode specification in the mouse, but suggested instead that *Sox9* controls adhesive properties and invagination of placodal cells in a cell-autonomous manner (Barrionuevo et al., 2008). The early arrest of inner ear development in this mouse model precludes investigation of an independent role of *Sox9* in the periotic mesenchyme in which it is also expressed. Here, we use a $Tbx18^{Cre}$ line to inactivate *Sox9* specifically in the mesenchymal compartment of the inner ear. We show that *Sox9* is not required for early condensation and compartmentalization of this tissue but for formation of the cartilaginous otic capsule in the outer compartment. Inactivation of *Sox9* results in profound defects in expansion and differentiation of otic fibrocytes and cochlear duct morphogenesis revealing crucial tissue interactions between mesenchymal and epithelial compartments in the developing cochlea *in vivo*.

Material and methods

Mouse lines and embryo preparation

For the generation of a *Cre* Knock-in allele of *Tbx18* ($Tbx18^{Cre}$, $Tbx18^{tm4(cre)Akis}$) a targeting vector was constructed to insert into the *Tbx18* locus a *Cre* ORF followed by a *PGK-neo* cassette flanked by *FRT* sites. After homologous recombination the ATG codon of *Cre* replaced the endogenous *Tbx18* start codon, and a 0.8 kbp genomic fragment spanning from the ATG to a 3′-NotI site was deleted (Supplemental Fig. 1). The targeting vector was confirmed by restriction mapping and sequencing and linearized for electroporation into 129/SvImJ ES cells. 24 h after electroporation, selection of transgenic clones started by addition of 125 µg/ml G418 to the medium. Surviving colonies were expanded and genotyped by Southern blot similar to the generation of *LacZ* and *Gfp* knock-in alleles of *Tbx18* (Bussen et al., 2004; Wiese et al., 2009). Two of five verified ES clones with a homologous recombination event were microinjected into CD1 mouse morulae. Chimeric males were obtained and mated to a *FLPe* deleter line (*Tg(ACTFLPe)9205Dym/J*) (Rodriguez et al., 2000) to produce F1 animals with the *neo* cassette removed. $Tbx18^{Cre}$, $Sox9^{flox}$ ($Sox9^{tm1Gsr}$) (Kist et al., 2002) and $Rosa26^{LacZ}$ (*Gt(ROSA)26Sor*) (Soriano, 1999) mice were maintained on an NMRI outbred background. Embryos for *Sox9* expression analysis were derived from matings of NMRI wild-type mice. $Tbx18^{Cre/+}$;$Sox9^{flox/flox}$ (*Sox9KO*) mice were obtained from matings of $Tbx18^{Cre/+}$;$Sox9^{flox/+}$ males and $Sox9^{flox/flox}$ females. Single heterozygous littermates were used as controls for mutant embryos. $Tbx18^{Cre/+}$;$Rosa26^{LacZ/LacZ}$ mice were obtained from matings of $Tbx18^{Cre/+}$;$R26^{LacZ/+}$ males and $R26^{LacZ/LacZ}$ females. For timed pregnancies, vaginal plugs were checked in the morning after mating, noon was taken as embryonic day (E) 0.5. Embryos were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR. AKO1110 sense primer (5′-CAGATCTCGGGAGGCATCG-3′) and AKO1111 antisense primer (5′-ATTCTCCCACCGTCAGTACG-3′) amplified a 723 bp fragment from the $Tbx18^{Cre}$ allele. The oligos AKO0917/R1295 (5′-GCGAAGAGTTTGTCCTCAACC-3′), AKO0918/R523 (5′-GGAGCGGGAGAAATGGATATG-3′) and AKO0919/R26F2 (5′-AAAGTCGCTCTGAGTTGTTAT-3′) were used in a multiplex PCR and amplified a 500 bp fragment from the *Rosa26* wild-type allele and a 250 bp fragment from the *LacZ* allele, respectively. AKO0745 (5′-CCGGCTGCTGGGAAAGTATATG-3′), AKO0746 (5′-CGCTGGTATTCAGGGAGGTACA-3′), and AKO0747 (5′-CTCCGGTAGCAAAGGCGTTTAG-3′) amplified a 247 bp fragment from the *Sox9* wild-type allele and 419 bp/314 bp fragments from $Sox9^{flox}$/$Sox9^{del}$ alleles.

Details on PCR conditions are available on request.

Histological analysis and 3-D reconstruction

Embryos were embedded in paraffin wax, sectioned to 5 or 10 µm and stained with Hematoxylin and Eosin (5 µm) or Alcian blue and Eosin (10 µm). For Alcian blue staining deparaffinized and rehydrated sections were incubated for 30 min in freshly filtrated Alcian blue solution (pH2.5, 1 g Alcian Blue 8GX (Sigma) per 100 ml 3% acetic acid), washed in PBS and counterstained with Eosin. 3-D reconstructions of Alcian blue stained serial sections were performed according to published protocols (Soufan et al., 2003).

RNA in situ hybridization analysis

RNA in situ hybridization analysis on 10 µm paraffin sections was performed following a standard procedure with digoxigenin-labeled antisense riboprobes (Moorman et al., 2001). Probes used were as follows: *Aggrecan* (NM_007424, Pos. 205–1167), *Axin2* (Neidhardt et al., 2000), *Bmp4* (Jones et al., 1991), *Bsnd* (Birkenhager et al., 2001),