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*Barhl*1 is required for the differentiation of inner ear hair cell-like cells from mouse embryonic stem cells

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ABSTRACT

Inner ear hair cells are mechanoreceptors responsible for hearing. Pathogenic defects of hair cell-specific genes are one of the major causes of deafness. The BarH class homeobox gene *Barhl1* is a deafness gene expressed in developing hair cells, yet the role of *Barhl1* during hair cell development remains poorly understood. In the present study, we first established an *in vitro* differentiation system to efficiently obtain mouse embryonic stem cell (mESC)-derived hair cell-like cells. Subsequently, a mESC line carrying a targeted disruption of *Barhl1* was generated using CRISPR/Cas9 technology and subjected to the established *in vitro* hair cell differentiation protocol. Targeted disruption of *Barhl1* does not affect the induction of mESCs toward early primitive ectoderm-like (EPL) cells and otic progenitors but strongly inhibits the differentiation of hair cell-like cells. Using RNA-sequencing and bioinformatics, we further unravel the molecular mechanism underlying *Barhl1*-mediated hair cell development. Our data demonstrate the essential role of *Barhl1* during hair cell development and provide a basis for the treatment of *Barhl1* mutation-based deafness.

1. Introduction

Inner ear hair cells within the auditory sensory epithelium serve as mechanoreceptors for the conversion of sound stimuli into electrochemical signals (Kelly et al., 2012). Defects in the formation of hair cells or degeneration of these cells lead to permanent hearing loss for millions of people worldwide (Carey and Amin, 2006; Izumikawa et al., 2005). Gene defects are one of the major causes of hereditary deafness, for example, mutations in *Myo7a* (Tang et al., 2016) and *Myo15a* (Chen et al., 2016) are found to be associated with sensorineural hearing loss. In human, a large number of deafness-related genetic loci have been described, however, only about 100 deafness genes have been identified (Scheffer et al., 2015). Therefore, identification of deafness genes and elucidation of the molecular mechanisms related to deafness are of fundamental importance for the treatment of hearing loss.

Genes specifically expressed in hair cells are considered to be good candidates for deafness genes because they are likely to play specialized roles in hair cells, and thus the mutation of these genes is likely to result in hearing impairment. *Barhl1*, a mammalian homolog of the *Drosophila BarH1* and *BarH2*, encodes a BarH class homeodomain transcription factor (Bulfone et al., 2000). *Barhl1* is uniquely expressed in developing inner ear hair cells (Li et al., 2002) and may play an important role during hair cell development. Targeted deletion of *Barhl1* in mice has been previously shown to cause severe to profound hearing loss (Li et al., 2002), demonstrating that *Barhl1* is a deafness gene. However, little is known about the molecular function of *Barhl1* in developing hair cells which is critical for understanding the pathogenesis of *Barhl1* mutation-based deafness.

Stem cell-based therapy has now become a promising strategy to regenerate inner ear hair cells to restore the damaged auditory function. Pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are suitable cell sources for cell

Abbreviations: mESCs, mouse embryonic stem cells; iPSCs, induced pluripotent stem cells; CRISPR, clustered regularly interspaced short palindromic repeat; Cas9, CRISPR- associated protein 9; MEF, mouse embryonic fibroblast; EBs, embryoid bodies; EPL, early primitive ectoderm-like; EBMs, EPL cell aggregates; EGF, epidermal growth factor; FGF3, fibroblast growth factor 3; FGF10, fibroblast growth factor 10; IGF-I, insulin-like growth factor-1; RA, retinoic acid; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate buffer saline; HBSS, Hanks' balanced salt solution; EDTA, ethylene diamine tetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole; CDS, coding sequence; AP, alkaline phosphatase; qRT-PCR, quantitative real-time polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; GO, gene ontology; RSAT, regulatory sequence analysis tools; SEM, standard error of the mean; WT, wild type

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therapy and they have already been used to successfully generate hair cell-like cells (Chen et al., 2012; Costa et al., 2015; De Silva et al., 2006; Koehler et al., 2013; Li et al., 2003; Oshima et al., 2010; Ouji et al., 2012; Ouji et al., 2013) although the efficiency of hair cell formation needs to be improved. CRISPR/Cas9 technology, due to its advantages in the high efficiency and specificity of gene targeting, is widely used in gene-editing. Combining pluripotent stem cell technology together with CRISPR/Cas9 gene-editing provides an important strategy to unravel the pathogenesis of diseases including hereditary deafness. In the present study, we first developed an *in vitro* differentiation system that is efficient in generating inner ear hair cell-like cells from mESCs. We then generated a mESC line carrying a targeted disruption of *Barhl1* using CRISPR/Cas9 technology and subjected it to the established *in vitro* hair cell differentiation protocol. We found that targeted disruption of *Barhl1* strongly repressed the differentiation of hair cell-like cells without affecting the induction of EPL cells and otic progenitors from mESCs. Furthermore, using RNA-sequencing and bioinformatics, we identified a number of putative Barhl1 direct target genes in hair cells. Our data demonstrate the essential role of Barhl1 during hair cell development and provide a basis for the treatment of *Barhl1* mutation-based deafness.

2. Materials and methods

2.1. mESC cell maintenance and differentiation

mESCs (ES-D3, a kind gift from Professor Jianzhong Shao, College of Life Sciences, Zhejiang University, China) were cultured on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS) (Life Technologies), 0.1 mM non-essential amino acids (Life Technologies), 55 mM 2-mercaptoethanol (Life Technologies), 2 mM GlutaMAX™ supplement (Life Technologies) and 1000 U LIF/ml (Chemicon, Shanghai, China). The medium was changed every day. Cells were passaged every other day, at constant plating density of 3×10^4 cells/cm^2. MEFs were prepared from 13.5 day-old CF-1 mouse embryos according to the previously described procedures (Lenka, 2006) and all animal experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang University. MEF feeder cells were then mitotically inactivated by 10 μg/ml mitomycin C (Sigma-Aldrich) for 3 h before they were used for culture of mESCs (Oshima et al., 2010).

For spontaneous differentiation, mESCs were dissociated using 0.25% trypsin-ethylene diamine tetraacetic acid (EDTA) (Life Technologies) and MEF feeder cells were removed by pre-culturing the cells for 1 h allowing MEFs to attach. mESCs were then seeded on 6-well cell suspension plates (Corning Inc., Corning, NY, USA) at a density of 1×10^5 cells/cm^2 and cultured for 4 days in DMEM supplemented with 15% FBS, 0.1 mM non-essential amino acids, 55 mM 2-mercaptoethanol and 2 mM GlutaMAX™ supplement to form embryoid bodies (EBs). The medium was changed every day. Subsequently, the resultant 4-day EBs were plated into gelatin-coated 24-well plates (Corning Inc.) at 10 EBs per well and cultured for 14 days in the same medium. The medium was changed every other day during the 2-week EB outgrowth cultures.

A conditioned medium (MEDII) required for the formation of early primitive ectoderm-like cells from mESCs was prepared as following: Hep G2 cells were seeded at 5×10^4 cells/cm^2 in DMEM supplemented with 10% FBS and 1 mM GlutaMAX™ supplement. After 4 days culture, the medium was then collected and sterilized by filtration (Rathjen et al., 1999). For EPL cell formation, after removal of MEFs, dissociated mESCs were seeded on 6-well cell suspension plates at a density of 1×10^5 cells/cm^2 and cultured for 7 days in DMEM/F12 (Life Technologies) supplemented with 10% FBS, 0.1 mM non-essential amino acids, 55 mM 2-mercaptoethanol, 2 mM GlutaMAX™ supplement and 50% MEDII. In the presence of MEDII, EPL cell aggregates (EBMs) formed within 24 h, and the medium was changed every day.

For the generation of otic progenitors, EBMs were transferred into gelatin-coated 24-well plates at 10 EBMs per well and cultured for 14 days in DMEM/F12 supplemented with 1% N2 (Life Technologies), 2% B27 (Life Technologies, without vitamin A), 50 ng/ml fibroblast growth factor 3 (FGF3) (R&D Systems, Shanghai, China), 50 ng/ml fibroblast growth factor 10 (FGF10) (R&D Systems), 25 ng/ml epidermal growth factor (EGF) (Life Technologies), 10 ng/ml insulin-like growth factor-1 (IGF-I) (Life Technologies) and 50 ng/ml heparan sulfate proteoglycan (Sigma-Aldrich, St. Louis, MO, USA). The medium was changed every other day.

For the generation of hair cell-like cells, otic progenitors were dissociated using Accutase (Life Technologies), and seeded (at a density of 3.5×10^5 cells/cm^2) on top of mitotically inactivated embryonic chicken utricle stromal cells, in 24-well plates. Co-cultures were grown for 30 days using DMEM/F12 supplemented with 1% N2, 2% B27, 25 ng/ml EGF and 10^{-6} M all-trans retinoic acid (RA) (Sigma-Aldrich). The medium was changed every other day. Isolation and inactivation by mitomycin C of embryonic chicken utricle stromal cells were performed as previously described (Oshima et al., 2010).

2.2. Generation of a mESC line carrying a targeted disruption of Barhl1

A mESC line carrying a targeted disruption of *Barhl1* was generated using CRISPR/Cas9 technology. sgRNA was designed based on *Barhl1* coding sequence (CDS) using the CRISPR design tool (http://www.genome-engineering.org). The sgRNA sequence is: 5′-CAC CGG GCT TTT TCA GGC GCA CCG G-3′ and 5′-AAA CCC GGT GCG CCT GAA AAA GCC C-3′. Cas9 plasmid were constructed as following: the plasmid LentiCRISPRv2 (Addgene, Beijing, China) was digested by *Bsm*BI (Fermentas, Waltham, MA, USA) and then ligated with sgRNA by T4 DNA ligase (New England Biolabs, Ipswich, MA, USA).

For lentiviral production, Cas9 plasmid was co-transfected into HEK293T cells with the packaging plasmids pVSVg (Addgene) and psPAX2 (Addgene) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. At 6 h post-transfection, the medium was replaced with mESC medium. At 48 h and 60 h post-transfection, the lentiviral supernatants were harvested and filtered through a 0.45-μm syringe filter (Merck Millipore, Billerica, MA, USA), respectively. The resulting lentiviral supernatant supplemented with 8 μg/ml polybrene (Sigma-Aldrich) was used for infection.

24 h before the first lentiviral supernatant collection, mESCs were seeded on mitotically inactivated MEF feeder cells at a density of 3×10^4 cells/cm^2. At the time of infection, the culture medium was changed to the freshly prepared lentiviral supernatant. 12 h later, the lentiviral supernatant was applied for a second infection. 12 h after the second infection, cells were passaged and cultured in mESC medium. After 3 days of culture, these cells were then passaged again and cultured in mESC medium supplemented with 0.8 μg/ml puromycin for 5 days to enrich for cells targeted by CRISPR/Cas9. The medium was changed every day.

The resulting puromycin-resistant cells were collected and seeded into mitotically inactivated MEFs coated 96-well plates (Corning Inc.) at a density of 1 cell/well and cultured in mESC medium. After about 5 days of culture, the monoclones were formed and further expanded for PCR, TA cloning and sequence analysis to determine the genotype at the site targeted for mutation. Briefly, genomic DNA around the target site for CRISPR/Cas9 was amplified using Q5 high fidelity enzyme (New England Biolabs). The primers used for PCR are: 5′-GGT TGA GTT TCT TCA GGG AGC TGC C-3′ and 5′-TCA CCA CTG GCT CCT ACT ACA ACC-3′. The PCR conditions were 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and finally followed by 72 °C for 10 min. Subsequently, PCR product was incubated with Taq enzyme at 72 °C for 15 min and cloned into T-Vector pMD19 (TAKARA, Liaoning, China). Single clones were picked for sequencing using M13 universal primers. Sanger sequencing was performed by BioSune Biotech (Shanghai, China).