Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 307 (2007) 165–178
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Multiple roles of Notch signaling in cochlear development

Shinji Takebayashi [a,b], Norio Yamamoto [b], Daisuke Yabe [b], Hitoshi Fukuda [b,c], Ken Kojima [a], Juichi Ito [a], Tasuku Honjo [d,*]

[a] *Department of Otolaryngology–Head and Neck surgery, Graduate School of Medicine, Kyoto University, 54 Shogoinkawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan*
[b] *Department of Medical Chemistry and Molecular Biology, Graduate School of Medicine, Kyoto University, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606-8501, Japan*
[c] *Department of Neurosurgery, Graduate School of Medicine, Kyoto University, 54 Shogoinkawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan*
[d] *Department of Immunology and Genomic Medicine, Graduate School of Medicine, Kyoto University, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606-8501, Japan*

Received for publication 22 December 2006; revised 27 March 2007; accepted 26 April 2007
Available online 3 May 2007

Abstract

Notch signaling inhibits hair cell differentiation, based on studies on mice deficient in Notch signaling-related genes and its downstream genes. However, the precise mechanisms of this inhibition are unknown because it is difficult to control the timing and duration of the suppression of Notch signaling. Here, we developed a novel *in vitro* culture and analysis method for mouse fetal cochleae and examined the roles of Notch signaling by its reversible inhibition through the use of Notch signaling inhibitors of γ-secretase and TNF-alpha-converting enzyme. Notch inhibition with Notch signaling inhibitor treatment increases the number of cochlear hair cells, as observed in gene deletion experiments. We elucidated that this increase is regulated by the dichotomy between hair cells and supporting cells from common progenitors. We also propose other roles of Notch signaling in cochlear development. First, Notch signaling arrests the cell cycle of the cochlear epithelium containing putative hair cells and supporting cell progenitors because Notch inhibition with inhibitor treatment increases the number of 5-bromo-2′-deoxyuridine (BrdU)-positive cells that can differentiate into hair cells or supporting cells. Second, Notch signaling is required for the induction of Prox1-positive supporting cells. Third, Notch signaling is required for the maintenance of supporting cells.

Keywords: Cochlea; Hair cell; Supporting cell; Progenitor cell; Organ culture; Notch signal; γ-Secretase inhibitor; TACE inhibitor

Introduction

Approximately 10% of the world's population suffers from hearing loss. Cochlear hair cell damage is one of the most important causes of sensorineural hearing loss; currently, there are no available therapeutics for its treatment because mammalian cochlear hair cells as against avian hair cells can never regenerate spontaneously (Ryals and Rubel, 1988; Corwin and Cotanche, 1988). It is very important to elucidate the molecular mechanisms underlying hair cell development for application in regenerative medicine.

Cochlear hair cells are sensory epithelial cells that transduce mechanical sound signals into electrical signals recognized by auditory neurons by using mechanoelectrical transduction channels. Hair cells are precisely arranged to form one row of inner and three rows of outer cells. These hair cells are surrounded by several types of supporting cells.

During inner ear development, Notch signaling has been implicated in the induction of supporting cell fate rather than hair cell fate in the cochleae, based on studies on mice deficient in Notch signaling-related genes (Lanford et al., 1999; Brooker et al., 2006; Kiernan et al., 2001, 2005, 2006) and its downstream genes (Zheng et al., 2000; Zhang et al., 2000; Zine et al., 2001). By analyzing conditional knockout mice having different Notch ligand genes, recent studies have suggested that Notch signaling might also control the proliferation of the supporting cell population (Kiernan et al., 2005) and play several roles in the different developmental stages of the cochlear sensory epithelium (Brooker et al., 2006; Kiernan et al., 2006). However, the precise mechanisms by which Notch signaling controls such cell differentiation pro-

* Corresponding author. Fax: +81 75 753 9485.
E-mail address: honjo@mfour.med.kyoto-u.ac.jp (T. Honjo).

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.04.035

cesses in the inner ears are largely unknown. This is because it is difficult to elucidate the roles of Notch signaling at different developmental stages by the irreversible inactivation of Notch signaling using gene-targeting technology.

Notch signaling is activated through the binding of ligands, such as Jagged and Delta, to Notch receptors 1–4 (Tax et al., 1994; Fleming, 1998). This binding initiates the proteolytic cleavage of Notch receptors at their extracellular region by TNF-alpha-converting enzyme (TACE) (Brou et al., 2000) and at intramembranous regions by γ-secretase activity (De Strooper et al., 1999; Mizutani et al., 2001). This cleavage releases the intracellular domain of the Notch receptors (NICD) from the membrane (Schroeter et al., 1998). NICD enters the nucleus, forms a complex with the DNA-binding protein RBP-J, and activates its target genes, thereby controlling cell differentiation (Jarriault et al., 1995; Tamura et al., 1995; Honjo, 1996). Yamamoto et al. showed that the γ-secretase inhibitor could efficiently block Notch signaling in neonate mouse cochleae and induced hair cells in cochlear explant cultures. Here, we developed a novel *in vitro* culture and analysis method for mouse fetal cochleae and addressed the regulation of inner ear development by using Notch signaling inhibitors of TACE and γ-secretase, both of which are independently required for Notch activation (Fortini, 2002). One of the main advantages of using these types of small molecule inhibitors is that the inactivation of target signaling by these inhibitors is reversible unlike irreversible inactivation by gene targeting; further, this culture method is suitable for analyzing the multiple roles of Notch signaling systems.

Taking advantage of these features, we showed that the increase in hair cell number by the inhibition of Notch signaling was due to hair cell differentiation from progenitor cells at the expense of supporting cells. Prox1-positive immature supporting cells can differentiate into hair cells when Notch signaling is inhibited. Moreover, we clearly showed that Notch signaling played a role in arresting the cell cycles of cochlear epithelial cells to enable their differentiation into hair cells or supporting cells.

Materials and methods

Animals

Time-pregnant ICR mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All experiments were performed in accordance with the guidelines of the animal research committee, Graduate School of Medicine, Kyoto University.

Explant culture of cochleae

Inner ears were dissected from the heads of embryonic day (E) 12.5, 13.5, 14.5, or 17.5 embryos by using forceps under a LEICA MZ7.5 stereomicroscope (Leica Microsystems, Wetzlar, Germany). The cochlear epithelium was removed mechanically from the cochlear bone in PBS and cut at the hook region. Spiral ganglion neuron cells remained attached to the cochlear epithelium although most mesenchymal cells were removed. The entire cochlear epithelium with attached spiral ganglion neurons was placed in a 30-μl drop of type I rat collagen at a final concentration of 2.5 mg/ml (Sigma-Aldrich, St. Louis, MO, USA) in 10 mM HEPES buffer (Invitrogen, Carlsbad, CA, USA), 1 mM HCl, 1× MEM (Invitrogen), and 0.5% $NaHCO_3$ (Invitrogen). After waiting for the drop to gel for 30 min at 37 °C at 5% CO_2, we added 200 μl of DMEM supplemented with 6 mg/ml glucose (Wako, Osaka, Japan) and 0.6 mg/ml penicillin G (Nacalai Tesque, Kyoto, Japan). The culture was incubated at 37 °C in a humidified 5% CO_2 atmosphere. The culture medium was replaced every day. *N*-[*N*-(3,5-Difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester (DAPT; EMD Biosciences, San Diego, CA, USA) was used as a γ-secretase inhibitor and *N*-(*R*)-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-naphthylalanyl-L-alanine-2-aminoethyl amide (TAPI-1; EMD Biosciences) or *N*-(*R*)-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-*t*-butyl-alanyl-L-alanine-2-aminoethyl amide (TAPI-2; EMD Biosciences) was used as a TACE inhibitor to inhibit Notch signaling. In a (+)-5-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) experiment, the culture medium was supplemented with BrdU at a final concentration of 3 μg/ml. DAPT, TAPI-1, or BrdU was dissolved in DMSO (Sigma-Aldrich); the final DMSO concentration in the culture medium was less than 0.1%. The control samples were supplemented with the same concentration of DMSO as the drug-treated samples.

Immunohistochemistry

Cultured cochleae surrounded with collagen were fixed at 4 °C for 30 min in 4% paraformaldehyde (Nacalai Tesque) after washing with PBS. For whole mount immunohistochemistry, whole cochleae were permeabilized with 0.2% Triton X in PBS for 1 h, blocked with 1.5% goat serum for 1 h, and then immunostained with primary and secondary antibodies. For whole mount immunohistochemistry performed using anti-BrdU antibodies, antigen retrieval was accomplished by incubation in 2 M HCl for 30 min at 37 °C before blocking. The immunostained sensory cochlear epithelium was removed from the whole cochleae under a LEICA MZ FLII fluorescence stereomicroscope (Leica Microsystems), placed in the aqueous mounting medium Permafluor (Beckman Coulter, Fullerton, CA, USA) on a slide glass, and covered with a coverglass. For frozen sections, the fixed cochleae were immersed in 30% sucrose at 4 °C overnight after fixation and then the surrounding collagen was removed. The whole cochleae were placed in OCT compound (International Medical Equipment, San Marcos, CA, USA) such that the basal turn of the cochleae were arranged in the same direction under a LEICA MZ7.5 stereomicroscope and preserved at −80 °C. Frozen sections were cut at 10 μm, mounted on slide glasses, and immunostained. Antigen retrieval was performed by incubation in 10 mM sodium citrate buffer (pH 6.0) with 0.5% Tween 20 for 20 min at 95 °C. For immunohistochemistry performed using biotinylated secondary antibodies, the sections were incubated for 30 min in absolute methanol containing 1% H_2O_2 to inhibit endogenous peroxidase activity. The sections were permeabilized, blocked, and immunostained as in the case of whole mount immunostaining. An avidin-biotinylated enzyme complex (ABC) kit (Vector Laboratories, Burlingame, CA, USA) and a diaminobenzidine (DAB) substrate kit (Vector Laboratories) were used for DAB staining performed using the ABC method. Nuclei were counterstained with hematoxylin or TOTO3 (1:10 000; Invitrogen). The VECTASHIELD mounting medium (Vector Laboratories) was used as a mounting medium for fluorescence sections. The following are the primary antibodies used in the indicated dilutions for the sections: anti-myosin VI rabbit antibodies (1:800, provided by Dr. Tama Hasson; UCSD, CA, USA), anti-myosin VIIa rabbit antibodies (1:600, provided by Dr. Tama Hasson), anti-Prox1 rabbit antibodies (1:100; AngioBio, Del Mar, CA, USA), anti-p27 rabbit antibodies (1:100; Lab Vision, Fremont, CA, USA), anti-S100A1 rabbit antibodies (1:100; Lab Vision), anti-BrdU mouse antibodies (1:50; BD Biosciences, San Jose, CA, USA), and anti-NICD rabbit antibodies (1:80; Cell Signaling Technology, Danvers, MA, USA). The following secondary antibodies were used: Alexa Fluor 488 goat anti-rabbit IgG antibodies (1:400; Invitrogen), Alexa Fluor 594 goat anti-mouse IgG antibodies (1:250; Invitrogen), Alexa Fluor 594 goat anti-rabbit IgG antibodies (1:250; Invitrogen), Alexa Fluor 633 phalloidin (1:1000; Invitrogen), and biotinylated goat anti-rabbit IgG (H+L) antibodies (1:200; Vector Laboratories). The primary or secondary antibodies for whole mount immunohistochemistry were used at half the dilution that was used for the frozen sections. The samples for immunofluorescence were observed with a Leica TCS SP2 confocal microscope (Leica Microsystems). The sections stained by the ABC method were mounted in xylene and observed under a Leica DM5000B microscope (Leica Microsystems). The images were imported into Paintshop (Jasc Software, Inc., Eden Prairie, MN, USA), and saved as Tiff files.