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GENERATION OF INDUCED NEURONS BY DIRECT REPROGRAMMING IN THE MAMMALIAN COCHLEA

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Abstract—Primary auditory neurons (ANs) in the mammalian cochlea play a critical role in hearing as they transmit auditory information in the form of electrical signals from mechanosensory cochlear hair cells in the inner ear to the brainstem. Their progressive degeneration is associated with disease conditions, excessive noise exposure and aging. Replacement of ANs, which lack the ability to regenerate spontaneously, would have a significant impact on research and advancement in cochlear implants in addition to the amelioration of hearing impairment. The aim of this study was to induce a neuronal phenotype in endogenous non-neural cells in the cochlea, which is the essential organ of hearing. Overexpression of a neurogenic basic helix–loop–helix transcription factor, *Ascl1*, in the cochlear non-sensory epithelial cells induced neurons at high efficiency at embryonic, postnatal and juvenile stages. Moreover, induced neurons showed typical properties of neuron morphology, gene expression and electrophysiology. Our data indicate that *Ascl1* alone or *Ascl1* and *NeuroD1* is sufficient to reprogram cochlear non-sensory epithelial cells into functional neurons. Generation of neurons from non-neural cells in the cochlea is an important step for the regeneration of ANs in the mature mammalian cochlea.
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Key words: reprogramming, auditory neurons, cochlear non-sensory epithelial cells, regeneration, spiral ganglion, hearing.

INTRODUCTION

The potential of transcription factors in inducing somatic cells into differentiated cells has been known since the demonstration of *MyoD*'s capability to transform fibroblasts into muscle-like cells (Davis et al., 1987). More recent work has proven that transcription factors can help shuttle between differentiated cells: pancreatic exocrine cells derive from pancreatic endoderm, as do β-cells (Gu et al., 2002), and the basic helix–loop–helix (bHLH) transcription factor *neurogenin3*, in combination with *Pdx1* and *Mafa*, can efficiently convert pancreatic exocrine cells into β-cells *in vivo* (Zhou et al., 2008). Previously, using the transcription factors *neurogenin1* (*Neurog1*), *NeuroD1* and *SRY-related high-mobility-group box 2* (*Sox2*), we demonstrated that cochlear non-sensory epithelial cells were competent to develop as neurons as ectopic expression of these factors was sufficient to induce a neuronal phenotype (Puligilla et al., 2010). However, neuronal induction of non-sensory cells only occurred at embryonic stages, and even then the efficiency of induction was relatively low.

Cochlear non-sensory epithelial cells, mechanosensory hair cells, their supporting cells and auditory neurons (ANs) develop from the otic placode (Rubel and Fritzsch, 2002). Cochlear hair cells are innervated by the bipolar ANs, which are responsible for conveying auditory signals from hair cells to the brain. Loss of auditory hair cells or ANs in the inner ear is the leading cause of congenital and acquired hearing impairment (Rubel and Fritzsch, 2002). For patients who do not have residual hearing, a cochlear implant, which directly stimulates ANs electrically, has been the only solution to restore their hearing. So far, no biological therapy to treat sensorineural hearing loss has been available mainly due to the lack of regeneration capacity in the adult inner ear. While the loss of ANs has been considered secondary to hair cell loss, an increasing body of evidence clearly indicates that ANs can degenerate as a result of noise exposure and aging while hair cells remain intact (Kujawa and Liberman, 2009; Lin et al., 2011; Engle et al., 2013;

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Abbreviations: ANs, auditory neurons; bHLH, basic helix–loop–helix; DIV, days *in vitro*; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FBS, fetal bovine serum; GER, greater epithelial ridge; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; LER, lesser epithelial ridge; Map2, microtubule-associated protein 2; SE, sensory epithelium; SNAP25, synaptosomal-associated protein-25; Sox2, SRY-related high-mobility-group box 2; TTX, tetradotoxin.

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Furman et al., 2013; Sergeyenko et al., 2013). Age-related primary degeneration of ANs in human temporal bones may contribute to the decline in hearing-in-noise performance (Makary et al., 2011). Moreover, several genetic mutations result in primary loss of ANs: a mutation in mitochondrial DNA, most commonly an A→G mutation at locus 3243, causes degenerative changes in ANs while the organ of Corti is generally spared (Nadol and Merchant, 2001; Takahashi et al., 2003), and mutations in the TIMM8A gene, which causes mitochondrial dysfunction, cause primary degeneration of ANs with the majority of the organ of Corti remaining intact (Bahmad et al., 2007). Therefore, inducing the regeneration of ANs in a damaged ear has significant implications for future advances in the biological treatment of deafness.

Recently, combinatorial expression of neural-lineage-specific transcription factors, including the bHLH transcription factor *Ascl1* (*Mash1*), was shown to directly convert embryonic and postnatal fibroblasts into neurons (Vierbuchen et al., 2010; Pang et al., 2011). Furthermore, *Ascl1* alone was sufficient to induce neurons from mouse fibroblasts (Vierbuchen et al., 2010), and *Ascl1* was directly linked to the expansion of neural progenitors, later cell cycle exit and neural differentiation (Castro et al., 2011). Based on these findings and the need to induce neurons at high efficiency in postnatal and adult inner ears, we investigated the neurogenic transcription factor, *Ascl1*, to examine its ability to directly convert embryonic and postnatal cochlear non-sensory epithelial cells into neurons. We show that *in vitro* overexpression of *Ascl1* in the non-sensory epithelial cells of both embryonic and postnatal mouse cochlea is sufficient to generate induced neurons at high efficiency, as defined by morphology, expression of neuronal markers, synaptic proteins and the generation of action potentials.

EXPERIMENTAL PROCEDURES

Experimental animals

Pregnant CD-1 and CBA/CaJ mice were obtained from the Charles River Laboratories. Embryonic day 13.5 (E13.5) and postnatal day (P1, 5, 10 and 20) mice were used to perform gain-of-function experiments, immunohistochemistry and electrophysiological recordings. All procedures involving animals were approved by the Sunnybrook Research Institute Animal Care and Use Committee, being conformed to the guidelines set out by the Canadian Council on Animal Care, the University of California San Diego Institutional Animal Care and Use Committee, and the Rutgers University Institutional Review Board for the Use and Care of Animals.

DNA constructs

The *pCIG.nucEGFP.Ascl1* expression vector and the control *pCIG.nucEGFP* vector were kindly provided by S. Pons (Institute for Biomedical Research of Barcelona) (Alvarez-Rodriguez and Pons, 2009). A *pCLIG-NeuroD1* expression vector was kindly provided by R. Kageyama (Kyoto University, Kyoto, Japan) (Inoue et al., 2002). The pCLIG vector uses a *CMV-IE enhancer/chicken β-actin promoter* and an *Internal Ribosomal Entry Site* (*IRES*) to drive the expression of *NeuroD1* and *EGFP* as independent transcripts. The *pCIG.nucEGFP* vector and the *cytoplasmic.RFP* vector (Clontech) were used as control vectors.

Electroporation of cochlear explant cultures

Cochlear explants were dissected at E13.5, P1, P5, P10 and P20. Cochlear explants at E13.5, P1 and P5 were electroporated as described previously (Jones et al., 2006; Dabdoub et al., 2008). In brief, the cochlear disk was oriented perpendicular to the surface of the Petri dish in a 10-μl drop of PBS containing 1-μg/μl DNA of the specific expression vector(s) or a control vector. The two electrodes were placed on opposite sides of the disk and the negative electrode was located facing the luminal surface of the sensory epithelium (SE) (Fig. 1A). For cells to be transfected with multiple expression vectors, equal concentrations of the desired expression plasmids (1.0-μg/μl for each vector) were electroporated (Fig. 1E) (Ono et al., 2009; Masuda et al., 2012). A ECM830 square wave electroporator (BTX Harvard Apparatus, Holliston, MA, USA) was used for electroporation. The following parameters were used: 27 V, 30-ms duration, 100-ms interval, 9 pulses per cochlea. For electroporation of P10 and P20 cochlear explants, the apical portion of the cochlea was used. The cochlear disk was placed in an electroporation chamber with a 2-mm^2 electrode surrounded by a silicone rubber sheet, and a 10-μl drop of PBS containing 1-μg/μl DNA was applied in the chamber and the negative electrode was located facing to the luminal surface of the SE (Fig. 1B). The following parameters were used: 14 V, 30-ms duration, 100-ms interval, 8 pulses per cochlea. As soon as pulsing was finished, 50 μl of culture media was immediately added to the droplet. Following electroporation, cochleae were established as explants with the luminal surface of the SE facing upward on MatTek dishes (MatTek Corporation, Ashland, MA, USA) coated with a layer of Matrigel (1:20 dilution; BD Biosciences, San Jose, CA, USA). Explants were maintained at 35 °C for at least 6 days *in vitro* (DIV) in 150 μl of culture media with 10% fetal bovine serum (FBS) and processed by immunohistochemistry.

Immunohistochemistry

Cochleae from embryos and newborn pups were removed, isolated, and processed as whole mounts. Immunocytochemistry was performed on cochlear explant cultures as described previously (Dabdoub et al., 2008; Puligilla et al., 2010). Cells were labeled with primary antibodies against TuJ1 (1:500; Sigma, St.Louis, MO, USA), microtubule-associated-protein 2 (Map2; 1:300; Sigma, St.Louis, MO, USA), Prox1 (1:250; R&D Systems, Minneapolis, MN, USA), synaptosomal-associated protein-25 (SNAP25; 1:1000; Covance, Berkeley, CA, USA), Synapsin I (1:1000; Millipore, Billerica, MA, USA), Myosin 7a (1:100; Developmental Hybridoma Bank, Iowa City, IA, USA) Sox2 (1:250; Santa Cruz Biotechnology, Dallas, TX, USA), Myosin 6 (1:1000; Proteus

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