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MECHANISMS OF DEVELOPMENT

Cochlear stem/progenitor cells from a postnatal cochlea respond to Jagged1 and demonstrate that notch signaling promotes sphere formation and sensory potential

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ABSTRACT

Hair cells and supporting cells of the mammalian cochlea terminally differentiate during development. Recent *in vitro* evidence suggests the presence of hair cell progenitors in the postnatal cochlea. Phenotypic properties of these cells and factors that promote their ability to generate spheres in aggregate cultures have not been reported. We define an *in vitro* system that allows stem/progenitor cells harvested from the early postnatal cochlea to develop into spheres. These spheres contain Abcg2, Jagged1 and Notch1 positive progenitor cells that can divide and generate new hair cell-like cells, i.e. immunopositive for specific hair cell markers, including Myosin VI, Myosin VIIa, Math1 and ability to uptake FM1-43.

We demonstrate that reducing Notch signaling with a gamma secretase inhibitor decreases the number of spheres generated following treatment of the stem/progenitor cell cultures. Additionally, activation of Notch by an exogenous soluble form of a Notch ligand, i.e. Jagged1 protein, promotes sphere formation and the sensory potential of cochlear stem/progenitor cells. Our findings suggest that Notch1/Jagged1 signaling plays a role in maintaining a population of Abcg2 sensory stem/progenitor cells in the postnatal cochlea.

1. Introduction

Cochlear hair cells (HCs) are terminally differentiated cells that serve as mechanosensory receptor cells that convert sound stimuli into electric signals. These HCs are susceptible to damage from noise trauma, aging, and aminoglycoside ototoxicity. Although HC replacement occurs spontaneously in birds and lower vertebrates (Corwin and Cotanche, 1988; Ryals and Rubel, 1988), the mature mammalian cochlea does not regenerate new HCs (Chardin and Romand, 1995; Corwin and Oberholtzer, 1997; Stone et al., 1998). Therefore a loss of cochlear HCs is considered to be a major cause of hearing impairment.

In the mouse cochlea, proliferation, mitotic exit, and cell type specification are all tightly coordinated to generate a functional auditory receptor. The cochlea is first evident as

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an outgrowth of the ventral aspect of the murine otocyst by E11.5 at which time the stem/progenitors of HCs and supporting cells are in a mitotic phase. These progenitors exit the cell cycle between E12.5 and E13.5 (Ruben, 1967) to form a zone of non-proliferating cells within the lateral wall of the elongating cochlear duct (Chen and Segil, 1999). Synchronization of cell cycle exit is dependent on the expression of $p27^{Kip1}$, which remains highly expressed in differentiated supporting cells (Chen and Segil, 1999; Chen et al., 2002). After E14.5, a wave of differentiation begins in the basal portion of the cochlea that patterns the post-mitotic prosensory area into a characteristic mosaic pattern of HCs and supporting cells (Lim and Anniko, 1985; Kelley, 2006).

Stem/progenitor cells have been demonstrated in adult vestibular receptors (Li et al., 2003), but have not been identified in the mature cochlea. Recent studies (Doetzlhofer et al., 2004; Zhai et al., 2005; White et al., 2006; Savary et al., 2007) have reported the purification of a population of HC progenitors from the early postnatal mouse cochlea but the molecular factors responsible for promoting self-renewal and formation of cellular spheres have not been demonstrated.

Hair cell differentiation requires the expression of Math1, the mammalian atonal homologue (Bermingham et al., 1999; Zheng and Gao, 2000; Shou et al., 2003), and the proper patterning of the cochlea is governed in part by Notch-dependent cell–cell interactions (Lanford et al., 1999; Zine et al., 2000; Woods et al., 2004).

In other systems, Notch signaling has been reported to underscore neural stem cell self-renew and maintenance of the progenitor cell state. Upon ligand binding, Notch receptors are activated by serial cleavage involving members of the ADAM protease family and by intramembranous cleavage regulated by gamma secretase. Intramembranous cleavage causes translocation of the intracellular domain of Notch (NICD) to the nucleus, where it acts on downstream targets, e.g. *Hes1* and *Hes5* genes (Ohtsuka et al., 1999; Kadesch, 2000; Fre et al., 2005).

Abcg2 is a half-transporter also called Bcrp1, i.e. a member of the ATP-binding cassette (ABC) family of cell-surface transporter proteins. Unlike other ABC half-transporters, Abcg2 is expressed exclusively in the plasma membrane (Rocchi et al., 2000) and considered to be a universal marker of stem/progenitor cells (Bunting, 2002; Alison, 2003). Abcg2 is expressed by stem cells from several tissues with a pattern of distribution that supports the hypothesis that it maintains stem cells in an undifferentiated state (Zhou et al., 2001).

In the experiments reported in our study, we observed that dissociated epithelial cells from the sensory epithelium from the cochlea of a postnatal day-3 (P3) mouse posses the ability to form spheres in the presence of EGF and/or TGFα that are composed primarily of Abcg2 positive cells. In these spheres, a subset of hair cell-like cells arose from mitotic progenitors and these cells displayed molecular markers that characterize differentiated HCs.

Modulation of Notch signaling on spheres formation through either the loss or gain-of-functions experiments demonstrates the potential involvement of Jagged1/Notch signaling pathway in maintaining Abcg2 cochlear stem/progenitor cells and promoting their sensory potential after *in vitro* differentiation.

2. Results and discussion

The identification of HC progenitors in the postnatal mammalian cochlea (Doetzlhofer et al., 2004; Zhai et al., 2005; White et al., 2006; Savary et al., 2007) is of considerable interest because of their potential for replacing lost HCs after an injury to the organ of Corti. The evaluation of such potential requires characterization of phenotypic properties and cellular regulatory mechanisms of these progenitor cells.

We have used an *in vitro* system to characterize the conditions required for the isolation and culture of HC progenitors from early postnatal mouse cochlea. Visual inspection of the dissociated cochlear cells confirmed that the initial cultures contained no clusters of cells. Cultures just after plating did not immunostain for the presence of myosin VI (hair cell marker) indicating that the initial cell suspensions did not contain any mature preexisting HCs (data not shown). HCs from the initial preparation were excluded because they were either retained by the cell filtration strainers and/or killed during the enzymatic and mechanical cell dissociation procedure. In this *in vitro* system, Abcg2 positive progenitor cells formed spheres in non-adherent conditions in the presence of EGF/TGFα and generated hair cell-like cells when shifted to an adherent culture condition. These hair cell-like cells express several molecular markers that characterize differentiated HCs (i.e. myosin VI, myosin VIIa, math1, fimbrin and ability to uptake FM1-43). Furthermore, we demonstrate that modulating the Notch signaling pathway affects the formation of spheres from dissociated progenitor cells in a context dependent manner.

2.1. *Dissociated cells from P3 mouse cochlear epithelium form cellular spheres in response to EGF and/or TGFα*

Starting between 2 and 3 days *in vitro* (DIV) in the 'non-adherent' culture condition, cells seeded at low density in the presence of EGF and/or TGFα proliferated and formed multicellular floating spheres (Fig. 1A). Immunolabeling of these spheres after 6 DIV for Abcg2 (Bunting, 2002; Savary et al., 2007) showed that approximately 50% of the cells were positive for this epithelial progenitor cell marker (Fig. 1B–D) (100 cells counted per experiment, $n = 3$). A typical culture composed of approximately 10^4 seeded cells after 6 DIV without growth factors contained 4 ± 2.08 spheres. In comparison, there were significantly more spheres formed (i.e. 41.25 ± 3.50) when the same density of dissociated cells were maintained in EGF/TGFα supplemented medium ($p < 0.05$, *t*-test analysis) (Fig. 1E). We observed optimal sphere formation when cultures were maintained in medium supplemented with EGF/TGFα at a concentration of 20 ng/ml each. This effect of EGF/TGFα on sphere formation is consistent with previous studies that have implicated the EGF and TGFα family of growth factors in proliferation within sensory regions of mature utricles *in vitro* (Yamashita and Oesterle, 1995; Kuntz and Oesterle, 1998) and their effect on organ of Corti explants (Zine and de Ribaupierre, 1988).

To determine if dividing progenitor cells contribute to sphere formation, we incubated dissociated cell cultures with BrdU for 3 days beginning after 2 days post-plating. Immunostained spheres showed that $20.18\% \pm 6.89$ of the cells had