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Induction of differentiation of human embryonic stem cells into functional hair-cell-like cells in the absence of stromal cells

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ABSTRACT

Sensorineural hearing loss and vestibular dysfunction have become the most common forms of sensory defects. Stem cell-based therapeutic strategies for curing hearing loss are being developed. Several attempts to develop hair cells by using chicken utricle stromal cells as feeder cells have resulted in phenotypic conversion of stem cells into inner ear hair-cell-like cells. Here, we induced the differentiation of human embryonic stem cells (hESCs) into otic epithelial progenitors (OEPs), and further induced the differentiation of OEPs into hair-cell-like cells using different substrates. Our results showed that OEPs cultured on the chicken utricle stromal cells with the induction medium could differentiate into hair-cell-like cells with stereociliary bundles. Co-culture with stromal cells, however, may be problematic for subsequent examination of the induced hair-cell-like cells. In order to avoid the interference from stromal cells, we cultured OEPs on laminin with different induction media and examined the effects of the induction medium on the differentiation potentials of OEPs into hair-cell-like cells. The results revealed that the culture of OEPs on laminin with the conditioned medium from chicken utricle stromal cells supplemented with EGF and all-trans retinoic acid (RA) could promote the organization of cells into epithelial clusters displaying hair-cell-like cells with stereociliary bundles. These cells also displayed the expected electrophysiological properties.

1. Introduction

Hearing loss has become one of the most common disabilities in the world, and affects almost every age group. Although hearing loss may not be life threatening, it can greatly influence quality of life and social interactions, and has a significant financial impact on society (Cotanche and Kaiser, 2010; Oshima et al., 2010a,b). The inner ear is a highly specialized sensory organ with a complex structure, and has been referred to as the labyrinth (Bodmer, 2008). It contains hair cells arranged in a highly organized pattern, and is innervated by sensory neurons. Hair cells are sensory receptors located in the inner ear. In the human cochlea, hair cells die due to variety of reasons such as age-related deafness (presbycusis), high doses of ototoxic drugs (gentamycin, cisplatin, aminoglycosides, etc.), genetic disorders, infectious diseases, and exposure to high levels of noise (Bodmer, 2008; Cheng et al., 2005; Clark and Bohne, 1999; Fligor and Cox, 2004; Matsui and Cotanche, 2004; Suter and von Gierke, 1987). The loss of hair cells in higher vertebrates appears to be non-reversible and leads to permanent hearing loss (Pan et al., 2013). Therefore, restoration of mammalian hearing requires the regeneration of inner ear hair cells for restoring the function of the inner ear. In the past few years, stem cell-based therapeutic strategies for curing hearing loss have been developed. The generation of new hair cells from a renewable source of progenitors is the principal requirement for the development of a cell-based therapy within this sensory organ (Savary et al., 2007).

Embryonic stem cells (ESCs) are pluripotent cells capable of developing into cells of all the three germ layers (Odorico et al., 2001). With respect to hair cell regeneration, it was reported that

Abbreviations: hESCs, human embryonic stem cells; OEPs, otic epithelial progenitors; ONPs, otic neural progenitors; FGF3, fibroblast growth factor 3; FGF10, fibroblast growth factor 10; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; RA, all-trans retinoic acid; MEF, mouse embryonic fibroblast; KOSR, knockout serum replacement; RT-PCR, reverse transcription-polymerase chain reaction; DAPI, 4′,6-diamidino-2-phenylindole; SEM, scanning electron microscopy.

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murine embryonic stem cells (mESCs) could generate inner ear progenitors in vitro (Li et al., 2003). Ectodermal lineage cells from mESCs of transgenic *Math1*/nGFP mice were generated in another study using a stepwise method with otic-inducing growth factors (Oshima et al., 2010a,b). The generated otic progenitor cells had the capacity to develop into mechanosensitive sensory hair cells with the immature hair cell transduction currents. In addition to mESCs, there have been attempts to differentiate human ESCs (hESCs) into inner ear hair cells in the presence of growth factors. Chen et al. (2012) presented an induction protocol for differentiation of hESCs into otic progenitors. The otic progenitors were able to differentiate into hair-cell-like cells that displayed the expected electrophysiological properties. These hair cell-like cells displayed protrusions reminiscent of stereociliary bundles, but failed to mature fully into cells with typical hair cell cytoarchitecture.

The main goal of our study was to induce the differentiation of hESCs into the functional hair cells with stereociliary bundles responsive to voltage stimulation. In our studies, we used a two step-induction protocol of generating the functional hair cells from hESCs. We tested the effect of different substrates (laminin and chicken utricle stromal cells) in the process of differentiation. In order to avoid the interference of stromal cells, the otic epithelial progenitors (OEPs) induced from hESCs were cultured on laminin under different differentiation conditions, and the effects of these differentiation conditions on the differentiation of OEPs into hair-cell-like cells were examined. Our objective was to develop a culture method that may promote the differentiation of OEPs into the functional hair-cell-like cells with the expression of marker genes and the morphology of hair bundles under differentiation conditions in the absence of stromal cells. Furthermore, these hair-cell-like cells should be responsive to voltage stimulation, displaying functional properties of hair cells (Marcotti et al., 1999).

2. Materials and methods

2.1. Preparation of the embryonic chicken utricle stromal cells and the conditioned medium

Utricles were dissected from embryonic day 18 (E18) chicken and treated with DMEM/F12 (Invitrogen, Shanghai, China), supplemented with 0.5 mg/ml thermolysin (Sigma, Shanghai, China) at 37 °C for 40 min. The sensory epithelia were removed after adding DMEM/F12 supplemented with 10% FBS. The remaining pieces of the stromal tissue were transferred into a 3.5 cm culture dish containing 150 μl of PBS and 150 μl of pre-warmed 0.25% Trypsin/EDTA (Gibco, Shanghai, China), and incubated at 37 °C for 8 min in a CO_2 incubator. After addition of 400 μl DMEM/F12 supplemented with 10% FBS, the cells were gently resuspended and then cultured in a 10 cm culture dish until 80–90% confluency. At the first passage, cells were collected and filtered through a 70-μm strainer (BD Labware, Shanghai, China) to remove debris. Cells were passaged twice before using them for supporting the differentiation of inner ear hair cells. Prior to the differentiation experiment of inner ear hair cells, the chicken utricle stromal cells were seeded in gelatin-coated six-well dish (Corning, Shanghai, China), and cultured until 90% confluency. These cells were treated with 2 μg/ml mitomycin C in DMEM/F12 with 10% FBS for 3 h to arrest cell cycle, and washed five times with DMEM/F12 with 10% FBS before use.

For preparation of the conditioned medium, the chicken utricle stromal cells were seeded in gelatin-coated 15 cm culture dish (Corning) and cultured with Eagle's alpha minimum essential medium (α-MEM; Sigma) supplemented with 20% FBS (Gibco) and 50 mg/ml ampicillin. When the cells reached 90% confluency, the medium was replaced with 30 ml fresh medium. This medium was collected after 48 h, and used as the conditioned medium of chicken utricle stromal cells.

2.2. Induction of otic progenitors directly as monolayers

The hESC line H1, obtained from Prof. Xiao in College of Animal Science, Zhejiang University (Wu et al., 2008). These cells were cultured on inactivated mouse embryonic fibroblast (MEF) feeder cells in DMEM/F12 supplemented with 20% knockout serum replacement (KOSR; Gibco), 1% nonessential amino acids, 2 mM L-glutamine (Invitrogen, Shanghai, China), 0.1 mM 2-mercaptoethanol (Sigma, Shanghai, China), 50 mg/ml ampicillin, and 4 ng/ml of basic fibroblast growth factor (bFGF; Invitrogen).

Before induction of differentiation, the hESCs were dissociated into small clumps using collagenase IV (Invitrogen). These small clumps were further dissociated using 0.025% Trypsin–EDTA (Sigma). The tryptic digestion was terminated using a trypsin inhibitor, and the cell suspension was passed through a 100 μm cell strainer (BD Labware). The 100 μm strainer retained few clumps of two to three cells. Cells were plated at the density of 1×10^4 cm^{-2} into laminin-coated plastic (5 μg/cm^2; R&D systems, Shanghai, China). Cells were incubated in DMEM/F12 supplemented with N2 (1:100), B27 (1:50) (Invitrogen), FGF3 (50 ng/ml; Invitrogen), and FGF10 (50 ng/ml; Invitrogen) for 12 days. The medium was replaced with fresh medium every two days. During the first few days post-plating, a high level of cell death was detected. Hence, in the initial days, we added Y-27632 (Sigma, 10 μM) in the medium to decrease cell death. After 12 days of differentiation, most hESCs had differentiated into otic progenitor cells. There were two morphologically distinct types of otic colonies. One cell population showed a flat phenotype with a large amount of cytoplasm and formed epithelioid islands; these were identified as otic epithelial progenitors (OEPs). The second population had small cells with denser chromatin, and presented cytoplasmic projections; this population was identified as otic neural progenitors (ONPs) (Chen et al., 2012).

2.3. Separation of epithelial progenitor cells and induction of differentiation into hair-cell-like cells

To enrich OEPs, cells surrounding the epithelial colony were lifted with a quick incubation in Accutase (Invitrogen) at 37 °C for two or three min. Once colony edges started to curl, cells surrounding the epithelial colonies were rinsed off. A prolonged accutase step allowed the collection of epithelial colonies that remained attached.

For induction of OEPs to differentiate into hair-cell-like cells, we used two substrates: laminin from human fibroblasts (laminin, Sigma) and inactivated embryonic chicken utricle stromal cells. For the differentiation on inactivated embryonic chicken utricle stromal cells, OEPs were separated by Accutase (Invitrogen) at 37 °C, and cultured in 24-well dishes coated with the chicken utricle stromal cells for two to four weeks. The induction medium used for hair cell differentiation was DMEM/F12 supplemented with N2 (1:100), B27 (1:50), all-trans retinoic acid (RA, 10^{-6} M; Sigma), and EGF (20 ng/ml; Invitrogen). The induction medium was replaced with fresh medium every second day. For the differentiation on laminin, OEPs were cultured in 24-well dishes coated with laminin for two to four weeks. For differentiation on laminin, we tested three media conditions for hair cell differentiation: (1) the induction medium (DMEM/F12 supplemented with N2 (1:100), B27 (1:50), RA (10^{-6} M) and EGF (20 ng/ml); (2) the conditioned medium of chicken utricle stromal cells; (3) the conditioned medium supplemented with EGF (20 ng/ml) and RA (10^{-6} M). The medium was replaced with fresh medium every second day. Differentiated cells were analyzed using a combination of total RNA preparation followed by reverse transcription-polymerase chain reaction

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