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Efficient induction of inner ear hair cell-like cells from mouse ES cells using combination of *Math1* transfection and conditioned medium from ST2 stromal cells

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ABSTRACT

We sought to establish a more efficient technique for induction of inner ear hair cell-like cells (HC-like cells) from embryonic stem cells (ES cells) by using a combination of two previously reported methods; ST2 stromal cell-conditioned medium, known to be favorable for HC-like cell induction (HIST2 method), and ES cells with transfer of the *Math1* gene (Math1-ES cells). Math1-ES cells carrying Tet-inducible *Math1* were cultured for 14 days with doxycycline in conditioned medium from cultures of ST2 stromal cells following formation of 4-day embryoid bodies (EBs). Although each of the previously introduced methods have been reported to induce approximately 20% HC-like cells and 10% HC-like cells in their respective populations in EB outgrowths at the end of the culture periods, the present combined method was able to generate approximately 30% HC-like cells expressing HC-related markers (*myosin6*, *myosin7a*, *calretinin*, *α9AchR*, *Brn3c*), which showed remarkable formation of stereocilia-like structures. Analysis of expressions of marker genes specific for cochlear (*Lmod3*, *Emcn*) and vestibular (*Dnah5*, *Ptgds*) cells indicated that our HIST2 method may lead to induction of cochlear- and vestibular-type cells. In addition, continuous *Math1* induction by doxycycline without use of the HIST2 method preferentially induced cochlear markers with negligible effects on vestibular marker induction.

1. Introduction

Inner ear hair cells (HCs), primary transducers for perception of sound and balance, are not regenerated in mammals once they are lost (Hawkins et al., 1976; Schacht, 1986), thus replacement using various medical strategies, such as gene or cell therapy, is required to improve hearing ability (de Felipe et al., 2011; Qi et al., 2014; Hu and Ulfendahl, 2013; Okano and Kelley, 2012). Forced expression of the transcription factor *Math1* (also known as *Atoh1*) *via* viral infection has been reported to generate new HCs *in vivo* (Kawamoto et al., 2003; Zheng and Gao, 2000; Staecker et al., 2014; Husseman and Raphael, 2009), while induction of HC-like cells from various stem cell sources has also been achieved using novel methods (McLean et al., 2016; Elbana et al., 2015; Hartman et al., 2015; Bramhall et al., 2014) and applied as translational therapy for individuals with hearing loss (Li et al., 2004; Oiticica et al., 2010; Xu et al., 2016; Barboza et al., 2016; Jongkamonwiwat et al., 2010).

Among several candidate cell sources, including neural stem cells (NSCs) from adult brain tissues (Ito et al., 2001), mesenchymal stem cells from bone marrow (Jeon et al., 2007), and olfactory precursor cells (Doyle et al., 2007), embryonic stem (ES) (Rathjen and Rathjen, 2001) and induced-pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006) are considered to be particularly promising because of their ability for self-renewal and pluripotency. Induction of inner ear HCs from ES and iPS cells was first reported by Oshima et al.

Abbreviations: HC, hair cell; ES cell, embryonic stem cell; HIST2, HC induction method using ST2-CM; CM, conditioned medium; Math1-ES cells, ES cells with transfer of the *Math1* gene; EB, embryoid body; NSC, neural stem cell; iPS cell, induced-pluripotent stem cell; Dox, doxycycline; ES-M, ES cell medium in the absence of LIF; LIF, leukemia inhibitory factor; SEM, scanning electron microscopy; FACS, fluorescence-activated cell sorter; Grp, gastrin releasing peptide; Lmod3, leiomodin3; Emcn, endomucin; Dnah5, dynein axonemal heavy chain 5; Ptgds, prostaglandin D2 synthase; SC, supporting cell; ED, embryonic day.

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(2010), though their efficiency for induction of HCs was found to be low, accounting for only around 1% to 2% of the total cell population. Recently, an elegant protocol for generation of inner ear organoids from mouse ES cells using a 3D culture method was reported, in which chemically defined culture medium with conditions mimicking the developmental environment of the inner ear was used under a 3D setting (Koehler et al., 2013; Liu et al., 2016; DeJonge et al., 2016). Although this 3D culture method does not require use of exogenous tissue or undefined medium components, several steps are needed and the main products are vestibular sensory organ-like structures.

We recently reported *in vitro* induction of HC-like cells from mouse ES cells using ST2 stromal cell conditioned medium (ST2-CM), termed the HIST2 method, which consists of only two steps, embryoid body (EB) formation for 4 days and subsequent EB outgrowth culture for 14 days in ST2-CM (Ouji et al., 2012). That method induced approximately 20% of established EB outgrowths to produce HC-like cells. In addition, we reported that ES cells carrying Tet-inducible *Math1* (Math1-ES cells) showed a high ability to differentiate into HC-like cells even in unconditioned normal medium, as those comprised approximately 10% of the cells in EB outgrowths grown in the presence of doxycycline (Dox) (Ouji et al., 2013). In the present study, we used the HIST2 method in combination with Math1-ES cells. As expected, up to 30% of the cultured cells were found to express HC-related markers, including *Math1*, *myosin6*, *myosin7a*, *calretinin*, *α9AchR*, and *Brn3c* (also known as *Pou4f3*), with remarkable formation of stereocilia-like structures.

2. Materials and methods

2.1. Cells

To produce Math1-ES cells, a Math1 gene expression-controllable ES cell line was exposed to doxycycline (Dox), then transfected using the Tet-On gene expression system (BD), as previously described (Ouji et al., 2013). Math1-ES cells were derived from EB3 cells (a kind gift from Dr. Hitoshi Niwa, RIKEN CDB, Kobe, Japan) and carried the blasticidin S-resistant selection marker gene driven by the Oct-3/4 promoter (active in undifferentiated state) (Niwa et al., 2000; Nishimura et al., 2003). Four Math1-ES clones, termed Math1-ES1, Math1-ES3, Math1-ES5, and Math1-ES6, used in our previous study (Ouji et al., 2013) were examined. Each of these clones demonstrated similar results in the following experiments, thus representative results from Math1-ES1 are presented. Cells were maintained in gelatin-coated dishes without feeder cells in Dulbecco's modified Eagle's medium (DMEM; Wako) supplemented with 10% FBS (GIBCO, Invitrogen, Carlsbad, CA), 0.1 mM 2-mercaptoethanol (Sigma), 0.1 mM nonessential amino acids (GIBCO), 1 mM sodium pyruvate (Sigma), and 1000 U/ml of leukemia inhibitory factor (LIF; Wako, JAPAN). For the stromal cell line, ST2 cells were obtained from BRC (RIKEN Cell Bank, BioResource Center, Tsukuba, Japan) and maintained in DMEM supplemented with 10% FBS (Sigma). Conditioned medium (CM) was collected from ST2 cells cultured with ES cell medium in the absence of LIF (ES-M). Briefly, confluent cells in 100-mm plates were washed with PBS and cultured with fresh ES-M. After 48 h, supernatants were collected, centrifuged for 5 min, and filtrated with a 0.22-μm syringe membrane filter (Miilipore, Billerica, MA), then used as conditioned medium (ST2-CM).

2.2. In vitro hair cell differentiation procedure

Differentiation of undifferentiated Math1-ES cells into HCs was performed using the procedure shown in Fig. 1A. Briefly, Math1-ES cells were dissociated by trypsin and cultured in hanging drops to form embryoid bodies (EBs) (Keller, 1995). The cell density of each drop was 500 cells per 20 μl of ES-M. After 4 days, EBs in hanging drops were collected and plated in plastic 100-mm gelatin-coated dishes (20 EBs per dish) in ES-M or ST2-CM, with or without Dox, then allowed to attach to form outgrowths. Half of the culture medium was changed to new medium every 2 days during the 2-week EB outgrowth culture period.

2.3. Quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA (1 μg) was extracted from cultured cells using TRIzol reagent (Invitrogen). Reverse transcription and qPCR were performed with a SYBR PrimeScript RT-PCR kit II (TaKaRa), according to the manufacturer's instructions, using primers purchased from TaKaRa Bio Inc. (see Supplementary Table S1). The amount of target gene PCR products were calculated relative to the internal control (β-actin), then compared between the experimental and control groups using the ΔΔCT method.

2.4. Immunocytochemistry

Immunofluorescence analysis was performed using a standard protocol. Briefly, cells were fixed in 4% paraformaldehyde, then cellular membranes were permeabilized with 0.1% Triton X-100 in PBS containing 1% BSA (TPBS). The primary antibodies used and dilutions in TPBS were as follows: anti-Brn3c, 1:400 (Covance, Emeryville, CA); anti-myosin6, 1:100 (Santa Cruz, Santa Cruz, CA); anti-α9AchR, 1:100 (Santa Cruz); Lmod3, 1:100 (Santa Cruz); and Dnah5, 1:100 (Santa Cruz). Following incubation overnight at 4 °C and washing with TPBS 3 times, AlexaFluor 488 or 546 conjugated anti-goat, anti-rabbit, or anti-mouse secondary antibodies (Molecular Probes, Invitrogen) were used to detect primary antibodies. All nuclei were stained with DAPI (Dojin). After incubation for 1 h at room temperature and washing with TPBS 3 times, fluorescence was detected with a laser scanning confocal imaging system (FLUOVIEW FV1000, Olympus, Tokyo, Japan), then the images were modified using Photoshop CS (Adobe Systems, San Jose, CA) to selectively detect intensely fluorescent cells.

2.5. Scanning electron microscopy (SEM)

After undergoing differentiation for 18 days, Math1-ES cells were replated on membrane filters (BD Bioscience, Bedford, MA). Following overnight cultivation, the membranes were washed with PBS and replaced with 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 45 min. The buffer was removed and the membranes were post-fixed with 1% osmium tetroxide in buffer (60 min), then washed 3 times with fresh buffer (10 min each) followed by washing with distilled water (10 min), and dehydrated with an ethanol series and HMDS (Polysciences, Warrington, PA) for 5 min. Finally, they were coated with osmium and examined using a scanning electron microscope (S-4800, HITACHI, Hitachi, Japan).

2.6. Dye permeation experiment

Cells were exposed to FM1-43FX (5 μM, Invitrogen) for 10 s at room temperature and washed thoroughly with PBS, then fixed in 2% PFA and counterstained with DAPI (Hu and Corwin, 2007). Cells in randomly chosen fields ($n = 10$) were examined using confocal microscopy.

2.7. Statistical analysis

The fraction of immuno-positive cells among total cells was determined in a double-blind fashion by observations of approximately 300 cells in each of 10 randomly selected microscopic fields per experiment. Determinations were conducted 3 times for each experiment and significance was calculated using Student's *t*-test.