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Docosahexaenoic acid promotes differentiation of photoreceptor cells in three-dimensional neural retinas

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ABSTRACT

Retinal tissues generated from human pluripotent stem cells can be an excellent tool for investigating pathogenesis of retinal diseases and developing new pharmacologic therapies. Moreover, patient derived retinal tissues could allow for retinal transplantation therapy for degenerative retinal diseases. However, obtaining retinal tissues with matured photoreceptor outer segments, which are essential for photoreceptor functions, is currently challenging. Here we investigated the effects of docosahexaenoic acid (DHA) for maturation of photoreceptor outer segments at the late stage and visual chromophore analog, 9-*cis*-retinal for the early stage of differentiation to three-dimensional (3D)-retinal tissues from human embryonic stem cells (hESCs), respectively. In the presence of DHA, differentiated 3D-retinal tissues demonstrated improved maturation of photoreceptor outer segments and increased number of photoreceptor cells compared with tissues without DHA. Increased mRNA expression of mature photoreceptor markers was additionally documented in retinal tissues cultured with DHA. Conversely supplementation with 9-*cis*-retinal failed to improve differentiation of retinal tissues perhaps due to chronic aldehyde toxicity. The current study demonstrated that the addition of DHA to culture medium can help promote differentiation of photoreceptor outer segments *in vitro* and utilization of this methodology may lead to future therapies for patients with blinding diseases.

1. Introduction

Pluripotent stem cells, which include embryonic stem cells (ESCs) (Sjogren et al., 2004) and induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007) are attractive sources of cells for regenerative medicine. Specific cell types differentiated from these cells can be used not only to advance cell-based therapies but also to investigate disease pathophysiology and to develop novel pharmacological therapies. Successful application of stem cells for these purposes are heavily dependent on both the ability of stem cells to differentiate into specific cell types and the final quality of differentiated cells (Morizane and Lam, 2015).

Several studies have demonstrated that ESCs/iPSCs can develop into a three-dimensional (3D) retinal tissue that resembles the embryonic vertebrate eye (Kuwahara et al., 2015; Nakano et al., 2012; Zhong et al., 2014). The retinal anlage first appears as the optic vesicle evaginating from the forebrain. Subsequently, its distal portion invaginates to form a two-walled cup-like structure, the optic cup, which develops into the outer (pigmented) and inner (neurosensory) layers of the retina (Fuhrmann, 2010). The formation of a 3D-retinal tissue requires a versatile floating culture in serum-free medium as embryoid body-like aggregates with quick aggregation (SFEBq culture) (Eiraku et al., 2011; Eiraku et al., 2008; Wataya et al., 2008). Additionally various signaling modulators are required for a 3D-retinal tissue differentiation, and they include fetal bovine serum (FBS), a Hedgehog agonist smoothened agonist (SAG), a Notch inhibitor (DAPT) and morphogenetic protein-4 (BMP4). Although several groups have succeeded in producing retinal cells and 3D-retinal tissues from ESC/iPSC, the maturation of photoreceptor cells, namely lengthening of the outer segment, is not sufficient. Since a key function of the photoreceptor cells is receiving light through molecular signaling processes in the outer segments, successful outer segment formation is essential.

Abbreviations: BMP4, bone morphogenetic protein-4; CRALBP, cellular retinaldehyde binding protein; DHA, docosahexaenoic acid; FBS, fetal bovine serum; FGF, fibroblast growth factor; hESCs, human embryonic stem cells; iPSCs, induced pluripotent stem cells; NR, neural retina; ONL, outer nuclear layer; RPE, Retinal pigmented epithelium; SFEBq, serum-free culture of embryoid body-like aggregates with quick aggregation; 3D, three-dimensional.

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Previous works have shown that trophic factors display synergistic protective effects on photoreceptors (LaVail et al., 1992; Ogilvie et al., 2000). DHA, the most abundant polyunsaturated fatty acid in photoreceptors (Fliesler and Anderson, 1983), acts as a trophic molecule for photoreceptors and is essential for survival of these cells *in vitro* (Rotstein et al., 1996). Indeed, treatment of DHA leads to a delay in the onset of apoptosis, increases differentiation and enhances opsin expression in isolated photoreceptor cells (Politi et al., 2001; Rotstein et al., 1998).

Visual cycle defects can lead to retinal degeneration including retinitis pigmentosa and Leber congenital amourosis (Van Hooser et al., 2000). The visual cycle, a series of biochemical reactions occur in the photoreceptor and retinal pigmented epithelium (RPE), functions to continuously regenerate photoreceptor visual pigments by supplying visual chromophore 11-*cis*-retinal, which is indispensable to maintain the health of the retina (Kiser et al., 2012). It has been shown that 9-*cis*-retinal can also function as visual chromophore of rhodopsin (Van Hooser et al., 2000; Yoshizawa and Wald, 1963). In particular impaired production of visual chromophore is associated with poor maturation of photoreceptor outer segments and early photoreceptor degeneration (Cideciyan et al., 2015; Cideciyan et al., 2009; Maeda et al., 2010; Maeda et al., 2009b). Furthermore, supplementation of the visual chromophore rescues retinal degeneration in animals and humans especially at the early stage of retinal development (Koenekoop et al., 2014; Maeda et al., 2006; Maeda et al., 2010; Maeda et al., 2009b).

In the present study, we investigated whether supplementation of DHA or visual chromophore analog, 9-*cis*-retinal, could promote maturation of photoreceptor outer segments and the early development of the retinal tissue during differentiation of 3D-retinal tissues from pluripotent stem cells, respectively.

2. Material and methods

2.1. Retinal differentiation from hESCs

2.1.1. Protocol 1 for testing effects of visual chromophore 9-cis-retinal

ESCs (H9) (Thomson et al., 1998) were used for differentiation to 3D-retinal tissues. Protocols used in this study are summarized in Supplemental Fig. 1. For SFEBq culture (Wataya et al., 2008), hESCs were dissociated into single cells in TrypLE Express (Gibco, Carlsbad, CA) containing 0.05 mg/ml DNase I (Roche, Basel, Switzerland) and 20 mM Y-27632 (Watanabe et al., 2007), and quickly re-aggregated using low-cell adhesion 96-well plates with U-bottomed conical wells (Nunc^{TM} Low Cell Binding Microplates; Thermo Fisher Scientific, Waltham, MA) (12,000 cells per well, 100 μl) supplemented with 20 mM Y-27632 under 5% CO2 at 37 °C. The differentiation medium is growth factor-free chemically defined medium (gfCDM) (Wataya et al., 2008) supplemented with 10% Knockout Serum Replacement (KSR, Gibco), while gfCDM contains 45% Iscove's modified Dulbecco's medium (IMDM, Gibco), 45% Hams F12 (Gibco), Glutamax (Gibco), 1% chemically defined lipid concentrate (Gibco), monothioglycerol (450 mM, Sigma-Aldrich), 100 U/ml penicillin and 100 mg/ml streptomycin. The day on which the SFEBq culture was started was defined as day 0. Recombinant human BMP4 (R&D, Minneapolis, MN) was added to the culture medium to the final 1.5 nM (55 ng/ml) on day 6, and the half of the medium was replaced with the same medium containing 1.5 nM BMP4 on day 9, 12 and 15. To induce RPE tissues within NR, NR epithelium generated from hESCs was subjected to two-step 'induction-reversal' culture under the following conditions. For the 'induction' culture, NR-containing aggregates generated from hESCs on day 18 were transferred from a 96-well plate to a 10-cm Petri dish (noncell adhesive), and further cultured in suspension for 6 days under 5% CO_2 conditions in DMEM/F12-Glutamax medium containing 1% N2 supplement, 3 mM CHIR99021, 100 U/ml penicillin and 100 mg/ml streptomycin. An addition of 5 mM SU5402 (FGFR inhibitor; Sigma-Aldrich) to the induction medium increased both efficiency and reproducibility of RPE induction after the reversal culture. For the 'reversal' culture, the floating aggregates with RPE-like thin epithelium on day 24 were cultured in suspension under 40% O_2/5% CO_2 conditions in NR-differentiation containing DMEM/F12-Glutamax medium (Gibco), 1% N2 supplement (Gibco), 10% fetal bovine serum (FBS), 0.5 mM retinoic acid (Sigma-Aldrich), 0.1 mM taurine (Sigma-Aldrich), 0.25 mg/ml Fungizone (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Kuwahara et al., 2015).

For the treatment with 9-*cis*-retinal, 9-*cis*-retinal (Sigma-Aldrich) was admixed into the culture medium at the final concentration of 10 μM in the presence of 30 μM recombinant cellular retinaldehyde binding protein (CRALBP) on day 30 (Supplemental Fig. 1A, arrow) (Maeda et al., 2013). Procedures involving 9-*cis*-retinal were performed under dim red illumination at 4 °C. The same treatment with 9-*cis*-retinal was also conducted with Protocol 2 (Supplemental Fig. 1B, arrow).

2.1.2. Protocol 2 for testing DHA effects

Until day 23, the culture condition was the same as Protocol 1. On day 24, the floating aggregates were cultured in suspension at 37 °C in a humidified 5% CO_2 incubator in DMEM/F12 (3:1) supplemented with 2% B27, 1 × NEAA, and 1% antibiotic–antimycotic, where they gradually formed 3D-retinal tissues. Thereafter, the medium was changed twice a week. For long-term suspension culture, the medium was supplemented with 10% fetal bovine serum (Gibco), 100 mM Taurine (Sigma-Aldrich) and 2 mM glutamate (GlutaMAX, Invitrogen) beginning on day 35, unless otherwise noted. To promote photoreceptor maturation, 3D-retinal tissues were supplemented with 1 mM all-*trans*-retinoic acid (RA) (Sigma-Aldrich) on day 56 and subsequently RA concentration was decreased to 0.5 mM on day 84 (Supplemental Fig. 1B) (Zhong et al., 2014). On day 103, the floating 3D-retinal tissues were supplemented with 40 μg/ml DHA (Sigma-Aldrich) conjugated to 50 μM BSA fraction V (Roche) in PBS (Supplemental Fig. 1B, arrow) (Yu et al., 2012).

We originally planned to examine effects of 9-*cis*-retinal and DHA using Protocol 2. However because supplementation of 9-*cis*-retinal reduced cell viability with Protocol 2 as described in the result, 9-*cis*-retinal were also tested with our older protocol, Protocol 1. Effects of DHA were not examined with Protocol 1.

2.1.3. Expression and purification of CRALBP

Human CRALBP was purified from *E. coli*. BL21 (DE) LysS with N-terminal His-tag. The human CRALBP cDNA is cloned insite of the pET19b vector with a 23 residue N-terminal containing His-tag (Crabb et al., 1998). The human CRALBP protein was purified as described previously (Bolze et al., 2014; Maeda et al., 2013; McBee et al., 2001). In brief, *E. Coli.* was cultured overnight with agitation (225 rpm) at 37 °C in 100 ml of LB medium (10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone) containing 100 μg/ml ampicillin. One percent overnight grown culture was inoculated to 1.6 l LB medium. The bacterial cultures were grown upto 0.8 optical density (OD) followed by induction with 1 mM IPTG for 4 h with low agitation (180 rpm) at 37 °C. After 4 h of induction, *E. Coli.* was harvested by centrifugation at 7000 rpm for 10 min. The pellets were resuspended in 30 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) containing 1 mg/ml lysozyme. The resuspended pellets were sonicated followed by centrifugation at 15,000 rpm for 30 min to remove the cell debris. The supernatant was incubated with 6 ml 50% Ni-NTA agarose (Invitrogen) for 1 h and was loaded into a column. The Ni-NTA agarose column