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SCIENCE DIRECT
Developmental Biology 289 (2006) 243 – 252
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Retinal stem/progenitor properties of iris pigment epithelial cells

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Received for publication 7 July 2005, revised 13 October 2005, accepted 14 October 2005

Abstract

Neural stem cells/progenitors that give rise to neurons and glia have been identified in different regions of the brain, including the embryonic retina and ciliary epithelium of the adult eye. Here, we first demonstrate the characterization of neural stem/progenitors in postnatal iris pigment epithelial (IPE) cells. Pure isolated IPE cells could form spheres that contained cells expressing retinal progenitor markers in non-adherent culture. The spheres grew by cell proliferation, as indicated by bromodeoxyuridine incorporation. When attached to laminin, the spheres forming IPE derived cells were able to exhibit neural phenotypes, including retinal-specific neurons. When co-cultured with embryonic retinal cells, or grafted into embryonic retina in vivo, the IPE cells could also display the phenotypes of photoreceptor neurons and Muller glia. Our results suggest that the IPE derived cells have retinal stem/progenitor properties and neurogenic potential without gene transfer, thereby providing a novel potential source for both basic stem cell biology and therapeutic applications for retinal diseases.

Keywords: IPE; Regeneration; Retinal neuron; Retinal stem/progenitor

Introduction

Neural stem cells are found in all vertebrates throughout embryogenesis; however, as more and more neurons differentiate, the population of neural stem cells dwindles. It is thought that only a few stem cells are left, in particular locations, in the adult vertebrates.

In some cold-blooded vertebrates, the eye has a population of retinal progenitor cells that persist throughout life, which generates new neurons in the ciliary marginal zone (CMZ) (Wetts et al., 1989; Raymond and Hitchcock, 1997; Perron et al., 1998; Reh and Levine, 1998; Hitchcock et al., 2004). In warm-blooded vertebrates, retinal histogenesis was thought to occur during the early stages of development. However, several potential sources of neural regeneration have been reported in the eyes of mammals and birds. Retinal stem cells have been identified in the ciliary body (CB) (Ahmad et al., 2000; Fischer and Reh, 2000; Tropepe et al., 2000) and postnatal retina (Yang et al., 2002; Engelhardt et al., 2004; Klassen et al., 2004; Zhao et al., 2005). Müller glial cells have the capacity to produce neurons when stimulated to proliferate following retinal injury (Fischer and Reh, 2001; Ooto et al., 2004). Neuronal transdifferentiation from the retinal pigmented epithelial (RPE) cells can occur during the fetal or embryonic stages in birds and mammals, but this capacity is lost during development (Reh and Pittack, 1995; Zhao et al., 1995). Recent studies have reported that adult mammalian RPE cells have certain neural progenitor properties but cannot transdifferentiate into retinal specific neurons (Amemiya et al., 2004; Engelhardt et al., 2005). It remains unknown whether retinal stem cell properties are related to the transdifferentiation potential of RPE cells.

The IPE has the same developmental origin as the RPE and the retina, locating in the most peripheral region. The plasticity of IPE cells in urodeles has been well known for a long time. In the newt, which has a high capacity for tissue regeneration, if the

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0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.10.035

lens is surgically removed from the adult eye, a structurally and functionally complete lens always regenerates from the dorsal margin of the IPE. This phenomenon of lens regeneration is the clearest and most representative example of transdifferentiation that occurs naturally in vertebrate adult tissues (Wolff, 1895; Eguchi, 1988; Tsonis and Del Rio-Tsonis, 2004).

To our knowledge, data on IPE plasticity in higher vertebrates are limited to the results of our previous studies on chicken eyes. We succeeded in isolating postnatal chicken IPE and maintaining it for long periods in vitro, and demonstrated that cultured IPE cells could transdifferentiate into lens cells under permissive culture conditions (Kosaka et al., 1998, 2004). Also, in a collaborative study, we reported the possibility of neural induction from adult mammalian iris tissue cells. In adult rats, whole iris tissue derived cells (a mixture of IPE and stroma) became immunoreactive for photoreceptor-specific antigen only with *Crx* gene transfer (Haruta et al., 2001). *Crx* is the homeobox gene that is specifically expressed in the photoreceptors of the mature retina and is crucial in photoreceptor development (Chen et al., 1997; Furukawa et al., 1997;). The same group also reported similar effects on rat iris and CB derived cells through the overexpression of *Otx* and *Crx* genes (Akagi et al., 2004). These findings raised the possibility that IPE cells have dormant cell plasticity, even in higher vertebrates. However, it remained unknown whether pure IPE cells devoid of stroma could display retinal/neural stem cell properties or whether they could differentiate into retinal specific neurons without gene transfer. In this study, we confirmed the retinal stem/progenitor properties of pure postnatal chicken IPE cells, which were isolated without any other cell types.

Materials and methods

Composition of media and growth factors

Dulbecco's modified Eagle's medium/nutrient mixture (DMEM) with 8% fetal calf serum (FCS) and serum-free medium that contained D-MEM/F12 and N2-supplement were obtained from Invitrogen (Carlsbad, CA). FGF2ST medium was prepared from: D-MEM/F12 medium supplemented with FGF2 (20 ng/ml) for 2-days before the FGF2 was removed. Human recombinant EGF, FGF8, FGF9, FGF17 and FGF18 were obtained from R&D Systems, Inc. (Minneapolis, MN). Human recombinant FGF2 and FGF8 were obtained from PeproTech EC, Ltd. (London, UK).

Antibodies

The working dilutions and sources of the antibodies used in this study included the following: mouse monoclonal antibody against β-tubulin III (TuJ1; 1:500; Covance, Princeton, NJ), glial fibrillary acidic protein (GFAP; 1:400; Sigma-Aldrich, St. Louis, MO), vimentin (1:500; YIEM, Roma, Italy), Pax6 (1:50; Development Studies Hybridoma Bank, University of Iowa, Iowa City, IA), oligodendrocyte marker O4 (1:150; Chemicon, Temecula, CA), PKC (1:150, Pharmingen, San Diego, CA), syntaxin (HPC-1; 1:1000, Sigma-Aldrich), rhodopsin (RET-P1; 1:10000, Sigma-Aldrich), iodopsin (1:1000; a gift from Dr. Shichida, Kyoto University, Kyoto, Japan), sheep polyclonal antibody against Chx10 (1:200, Exalpha, Watertown, MA) and rabbit polyclonal antibody against musashi (1:500, Chemicon). The following secondary antibodies were used: goat-anti-mouse IgG (Alexa Fluor 488, 594, 1:500, Molecular Probes, Eugene, OR), goat-anti-mouse IgM (Alexa Fluor 488, 1:500, Molecular Probes), goat-anti-rabbit IgG (Alexa Fluor 488, 594, 1:500, Molecular Probes), donkey anti-sheep IgG (Alexa Flour 488, 1:200, Molecular Probes).

Preparation of IPE and CB cells from chickens

White leghorn chickens (2 days after hatching) were used as the source of IPE cells. All animal experiments were approved by the Animal Research Committees of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Riken Kobe Institute, Japan. In order to prevent contamination of the CB tissue, an incision (nearly 0.5 mm from the iris margin) was carefully made around the peripheral circumference of the iris (Fig. 1). To confirm whether IPE cell growth occurred in all parts of the iris, the tissues were divided into four different areas (the posterior, anterior, dorsal and ventral iris) and cultured. The IPE sheets were separated from the stroma as described previously (Kosaka et al., 1998). Epithelial cells from CB tissue (CE) were also isolated using similar methods.

Spherical culture of IPE and CE cells

Primary IPE and CE cells isolated from chicken eyes and dissociated IPE cells derived from the adherent culture were incubated in a non-coated dish in D-MEM/F12 medium supplemented with N2 supplement and growth factors (40 ng/ml) as a rotation culture. To test the efficiencies of primary sphere formation, cell preparations derived from three eyes were inoculated in one dish. After 3 days, the total number of spheres was calculated by scoring the sphere numbers in 10 randomly selected 10 μl samples of culture medium. For the secondary sphere culture, single cells dissociated from the primary spheres were counted and used.

Clonal growth assay

Freshly isolated and dissociated IPE cells (200 cells) were labeled with Qdot nanocrystals (Qtracker 565 cell labeling kit, QUANTUMDOT, Hayeard, CA) and co-cultured with unlabeled IPE cells (1000 multiples). Whole sphere colonies were examined at many different focal planes using a confocal laser-scanning microscope (LSM510; Carl Zeiss, Heidelberg, Germany), and the fluorescent and Normarsky images were compared ($n = 3$).

Cultured spheres that had been grown for 3 days were supplemented with 10 μM bromodeoxyuridine (BrdU) for 2 h. BrdU labeling and detections were performed using a 5-bromo-2′-unidine labeling and detection kit I (Roche Diagnostics, Basel, Switzerland).

Neural induction of IPE derived spheres

To induce neuronal differentiation, spheres that had been cultured for between 3 and 7 days were plated onto laminin- or collagen-coated dishes at a low density (3–5 spheres/cm^2) in FGF2ST medium and cultured for 2 weeks.

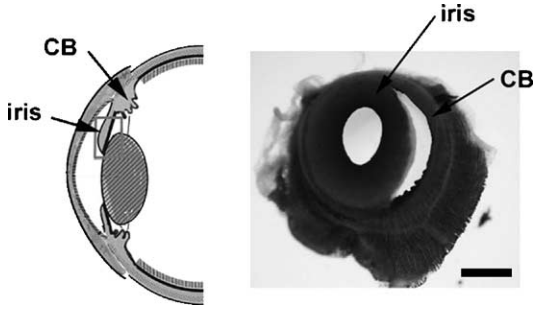

Fig. 1. Isolation of iris tissue from the eye. Nearly 0.5 mm from the ciliary margin, IPE tissue with stoma was cut. No part of the ciliary body (CB) contaminated the isolated iris tissue. Scale bar: 1 mm.